JBC Papers in Press. Published on October 10, 2003 as Manuscript M308230200

Riboflavin uptake and FAD synthesis in *Saccharomyces cerevisiae* mitochondria. *Involvement of the Flx1p carrier in FAD export.*

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Running Title: Involvement of the yeast Flx1p carrier in mitochondrial FAD export.

SUMMARY

We have studied the functional steps by which *Saccharomyces cerevisiae* mitochondria can synthesize FAD from cytosolic riboflavin (Rf). Riboflavin uptake into mitochondria took place via a mechanism that is consistent with the existence of (at least two) carrier systems. FAD was synthesized inside mitochondria by a mitochondrial FAD synthetase (EC 2.7.7.2), and it was exported into the cytosol via an export system which was inhibited by lumiflavin, and which was different from the riboflavin uptake system. To understand the role of the putative mitochondrial FAD carrier, Flx1p, in this pathway, an *flx1Δ* mutant strain was constructed. Coupled mitochondria isolated from *flx1Δ* mutant cells were compared to wild-type mitochondria with respect to the capability to take up Rf, to synthesize FAD from it and to export FAD into the extramitochondrial phase. Mitochondria isolated from *flx1Δ* mutant cells specifically lost the ability to export FAD, but did not lose the ability to take up Rf, FAD or FMN, and to synthesize FAD from Rf. Hence, Flx1p is proposed to be the mitochondrial FAD export carrier. Moreover, deletion of the *FLX1* gene resulted in a specific reduction of the activities of mitochondrial lipoamide dehydrogenase and succinate dehydrogenase, which are FAD-binding enzymes. For the flavoprotein subunit of succinate dehydrogenase (SDH-Fp) we could demonstrate that this was not due to a changed level of mitochondrial FAD or to a change in the degree of flavinylation of the protein. Instead, the amount of SDH-Fp was strongly reduced, indicating an additional regulatory role for Flx1p in protein synthesis or degradation.

INTRODUCTION

The mechanism by which mitochondria obtain their own flavin cofactors is an interesting point of investigation since FMN and FAD are mainly located in mitochondria, where they act as redox cofactors of a number of dehydrogenases and oxidases that play a crucial role in both bioenergetics and cellular regulation (for Reviews see 1, 2).

As far as mammalian mitochondria are concerned, we have demonstrated that in rat liver the main source of intramitochondrial flavin cofactors is riboflavin (Rf) [1] taken up from the cytosol. FAD

[1] The abbreviations used are: Rf, riboflavin; Fad1p, the product of *FAD1* gene; Flx1p, the product of *FLX1* gene; SCM, *Saccharomyces cerevisiae* mitochondria; DASPMI, 2-(4-dimetylaminostyryl)-N-methylpyridinium

synthesis occurs inside the organelle from imported Rf and mitochondrial ATP, consistent with the presence of a mitochondrial riboflavin kinase (EC 2.7.1.26) and an FAD synthetase (EC 2.7.7.2) (3, 4). Newly synthesized FAD can be either efficiently incorporated into newly imported apo-flavoproteins (5, 6) or can be exported into the outer mitochondrial compartments, where it is reconverted to Rf by FAD pyrophosphatase (EC 3.6.1.18) and FMN phosphohydrolase (EC 3.1.3.2) in a recycling pathway, i.e. the Rf-FAD cycle (4, 7). This novel mitochondrial pathway is assumed to play a central role in cellular Rf homeostasis and in flavoprotein biogenesis (5, 8).

The origin of flavin cofactors in yeast mitochondria is still controversially discussed. It has been reported that yeast mitochondria do not contain their own FAD synthetase activity, and that FAD is synthesized in the cytosol by Fad1p (9). Thus, mitochondria have been supposed to get their FAD from the cytosol via an FAD uptake system, encoded by the *FLX1* gene, a member of the mitochondrial carrier family (10). On the other hand, we have proposed that in yeast as well as in mammals Rf imported from the cytosol is the main source of mitochondrial flavin cofactors. Our observations implied a mitochondrial localization for both riboflavin kinase and FAD synthetase (11). Consistently, the mitochondrial localization of riboflavin kinase, encoded by the *FMN1* gene, has been recently confirmed by immunoblotting analysis (12).

In order to elucidate further the mechanism by which *Saccharomyces cerevisiae* mitochondria (SCM) can provide their own flavin cofactors, Rf uptake and metabolism were studied in intact organelles. Moreover, the activity of FAD synthetase has been determined in solubilized mitochondria. To clarify the role of the Flx1p carrier in this pathway, mitochondria isolated from *flx1Δ* mutant cells were compared to wild-type mitochondria with respect to the ability to take up Rf, to synthesize FAD and to export it out of the mitochondria. Moreover, the physiological role of Flx1p in maintaining the homeostasis of flavin cofactors has been studied as well as its influence on the amounts of FAD-binding enzymes.

iodide; PCR, polymerase chain reaction; D-AAO, D-amino acid oxidase; CS, citrate synthetase; PDC, pyruvate decarboxylase; LPDH, lipoamide dehydrogenase; SDH, succinate dehydrogenase; SDH-Fp, flavoprotein subunit

EXPERIMENTAL PROCEDURES

Materials. All reagents and enzymes were from Sigma, zymolyase from ICN and Bacto yeast extract from Difco. Mitochondrial substrates were used as Tris salts at pH 7.0. Solvents and salts used for HPLC were from Baker.

Yeast strain, media and growth conditions. The wild-type *S. cerevisiae* strain (EBY157A, genotype *MATα ura 3-52 MAL2-8^c SUC2 p426MET25*) used in this work is derived from the CEN.PK series of yeast strains and was obtained from P. Koetter (Frankfurt). Cells were grown aerobically at 28 °C with constant shaking in a semi synthetic liquid medium (3 g/l yeast extract, 1 g/l KH_2PO_4, 1 g/l NH_4Cl, 0.5 g/l NaCl, 0.5 g/l $CaCl_2 \cdot 2H_2O$, 0.6 g/l $MgCl_2 \cdot 6H_2O$, 20 mg/l uracil, 0.05 % glucose) supplemented with 2% ethanol or 2% glycerol as the carbon source. The pH of the medium was adjusted to 5.5 with HCl. Mitochondrial functionality in living cells was assessed by fluorescence microscopy**,** using the vital fluorescent dye 2-(4-dimetylaminostyryl)-N-methylpyridinium iodide (DASPMI) as described in (13).

Construction of an *flx1*Δ mutant strain. An *flx1*Δ mutant strain was constructed by using a modification of the polymerase chain reaction (PCR) targeting technique (14). A plasmid pUG6 (15) has been used as a template to generate by PCR a DNA molecule consisting of a *kanMX* marker cassette (conferring G418 resistance) flanked by short homology regions to the *FLX1* locus. For this purpose, appropriated oligonucleotides were constructed: S1-FLX1 [5'-ATGGTCGATCACCAGTGGACGCCACTGCAGAAGGAGGTTATCATCTCAGGTTTCGT ACGCTGCAGGTCGAC-3'] and S22-FLX1 [5'-TTTATCGTTTGATATTTACAAGAAATTAAATCTAT CATATAGCCTTTATGCATAGGCCACTAGTGGATCTG-3']. Short flanking homology regions allowed a specific recombination between wild-type chromosomal DNA containing the *FLX1* gene and linear DNA containing *kanMX*. The transformation of EBY157A strain was done by electroporation. The mutant yeast cells (EBY167A, genotype *MATα ura 3-52 MAL2-8^c SUC2, flx1*Δ*:: kanMX*) were selected for resistance to G418.

Construction of plasmid. The *FLX1* gene was cloned by PCR with a pair of primers designed to amplify a DNA fragment enclosing the complete *FLX1*-ORF with its 5' and 3' regulatory regions. The amplified fragment, cleaved with *Bgl*II and *Pvu*II, was cloned into the high-copy-number vector YEplac195 (16) with a *URA3* marker, resulting in plasmid YEpFLX1. The transformation of the EBY167A *S. cerevisiae* strain was done by electroporation.

Preparation of spheroplasts and mitochondria and oxygen uptake measurements. Spheroplasts were prepared using zymolyase and mitochondria were isolated according to (17)**.** Mitochondrial protein was determined according to (18). The intactness of mitochondrial inner membrane was checked measuring the

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of succinate dehydrogenase; RLM, rat liver mitochondria; PDH, pyruvate dehydrogenase.

release of the matrix enzyme fumarase, as in (19). Oxygen uptake measurements were carried out using a Gilson oxygraph as described previously (11).

Flavin uptake and metabolism. Mitochondria (0.1-0.2 mg protein), isolated from cells grown on glycerol-containing medium, were incubated at 2 °C in 500 µl of a medium consisting of 0.6 M mannitol, 50 mM Tris-HCl, pH 7, 1 mM $MgCl_2$. 1 min later, Rf, FAD or FMN were added. At appropriate time, the uptake reaction was stopped by rapid centrifugation. Rf, FMN and FAD content of supernatants and pellets were measured in aliquots (5-80 µl) of neutralized perchloric extracts by means of HPLC (Gilson HPLC system including a model 306 pump and a model 307 pump equipped with a Kontron Instruments SFM 25 fluorimeter and Unipoint system software), and corrected for endogenous flavin content, essentially as described in (4, 11). The amount of flavin actually taken up into the organelle was calculated after correction made for molecules present in the adherent space and/or non-specifically bound to the membranes, as described elsewhere (4, 20).

FAD synthetase activity assay. SCM (0.015-0.06 mg protein) were solubilized by treatment with Lubrol PX (0.3 mg/mg protein) for 15 min at 0 °C and pre-incubated for 1 min at 37 °C in 100 µl of a medium consisting of 50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$ in the presence of 5 mM sodium pyrophosphate. Then, FMN (10 µM) and ATP (5 mM) were added or not. After 2 min incubation, the reaction was stopped by boiling at 100 °C for 3 min followed by centrifugation at 14,000 g for 4 min at 4 °C. The amount of FAD present in the mitochondrial extracts was measured enzymatically, as described in (4, 11). Briefly, the amount of FAD was determined by revealing the reconstituted holo-D-amino acid oxidase (D-AAO) activity derived from the FAD binding to the apo-D-AAO, using D-alanine (25 mM) as substrate. The oxidation of NADH in the L-lactate dehydrogenase coupled reaction was followed photometrically at 340 nm by means of a Perkin Elmer λ19 spectrophotometer and its rate calculated as a tangent to the linear part of the progress curve. This rate was proven to be proportional to the FAD concentration. Calibration curves were obtained by using standard FAD solutions added to SCM. Corrections were also made to account for the inhibition due to FMN and ATP added to the assay.

Other enzymatic assays. Citrate synthetase (CS) and pyruvate decarboxylase (PDC) activities were measured as in (21, 22). Lipoamide dehydrogenase (LPDH) activity was assayed as reported in (23) and succinate dehydrogenase (SDH) activity was measured as the reduction rate of 2,6-dichlorophenol-indophenol in the presence of phenazine methosulfate, as reported in (24).

Western blotting. Proteins from SCM (0.04 mg) were separated by SDS-PAGE, according to (25) and transferred electrophoretically onto a nitrocellulose membrane using a trans-blot semidry electrophoretic

transfer cell (Sigma). The immobilized proteins were incubated overnight with a 1000-fold dilution of the specific polyclonal antibodies against FAD covalently bound to proteins (i.e. α-Fp antibodies containing antiserum raised in rabbits with the covalently flavinylated bacterial enzyme 6-hydroxy-D-nicotine oxidase, a kind gift from Prof. R. Brandsch, Freiburg, Germany) (26). Immunoreactive materials were visualized with the aid of a secondary alkaline phosphatase conjugated anti-rabbit IgG. Quantitative evaluations were carried by densitometric analysis using the Chemi Doc system (Bio-Rad).

UV-fluorescence analysis. Proteins from SCM (0.04 mg) were separated by SDS-PAGE, then the unstained gel was incubated for 1 h in 10% acetic acid and inspected on an UV-transilluminator system. Upon illumination with UV light, the flavinylated flavoprotein subunit of SDH (SDH-Fp) was visible because of the fluorescence of the covalently bound flavin. Protein bands on the gel were then stained with Coomassie Blue. Quantitative evaluations were carried using the Chemi Doc system (Bio-Rad).

RESULTS

Rf uptake and FAD synthesis in isolated intact SCM. To gain insight into the mechanism by which SCM can provide their own flavin cofactors, isolated mitochondria (from the wild-type strain EBY157A) were incubated with Rf in two different concentration ranges, i.e. 0-2.5 µM (Fig. 1A) and 0-30 µM (Fig. 1B). Then, the amounts of flavins in the acid-extractable fraction of both mitochondrial pellets and supernatants were measured by HPLC and compared with those of parallel samples in which no Rf was added. Experimental data were collected within the initial linear range of Rf uptake rates (i.e. 20 s incubation), and were corrected for adherent/bound vitamin as described in Experimental Procedures. Data were expressed as rates of flavin transport in dependence on Rf concentration.

FIG. 1

In the whole range of concentrations used (Figs. 1A and 1B), Rf transport was accompanied by FAD formation (see also 4, 11), which resulted in the export of FAD into the extramitochondrial phase. A residual FAD fraction was retained in the pellet. Conversely, no significant variation in FMN content was observed. Due to the rapid conversion of Rf into FAD (that could be prevented neither by adding ADP plus oligomycin, nor by changing the temperature and the time of incubation of mitochondria), a detailed study of the kinetics of Rf uptake was prevented. Hence, the Rf determined in the pellet under these conditions is a transient value, lower than the true amount of Rf

taken up. Thus, the time dependence of Rf increase in the pellet does not actually reflect a true Rf uptake rate.

In spite of the limitation imposed by this condition, the dependence of the apparent Rf uptake rate on vitamin concentration showed saturation characteristics (Fig. 1A). The sigmoidal feature of the curve probably depends on the combinations between transport and synthesis processes, rather than indicating true cooperativity. Nevertheless, data fitting was performed by means of Grafit software according to allosteric kinetics, and the resulting kinetic parameters, expressed as "pseudo" $S_{0.5}$ and V_{max}, were 1.2 µM and 28 pmol x min^{-1} x (mg protein)$^{-1}$, respectively. At the lower concentration of Rf used (0.2 µM, Fig 1A), FAD was synthesized with a rate of about 75 pmol x min^{-1} x (mg protein)$^{-1}$ and it was entirely retained in the pellet. At higher Rf concentrations (0.4-2 µM range, in Fig 1A), virtually no FAD could be detected in the pellet and the rate of FAD formation was described by the rate of FAD export, which reached a maximum of about 250 pmol x min^{-1} x (mg protein)$^{-1}$ at 2 µM.

When Rf concentration was increased up to 30 µM (Fig. 1B), the dependence of the apparent Rf uptake rate on the extramitochondrial vitamin concentration, showed again a sigmoidal shape. The "pseudo" $S_{0.5}$ and V_{max} values were 18 µM and 7750 pmol x min^{-1} x (mg protein)$^{-1}$, respectively. The rate of FAD formation starting from imported Rf increased with increasing external concentration of the vitamin, with a significant fraction of FAD remaining in the pellet. Under these conditions the rate of FAD export reached a maximum of about 600 pmol x min^{-1} x (mg protein)$^{-1}$at 10 µM Rf and it was inhibited at higher Rf concentrations.

In spite of the limitations described, these results strongly favor the existence of (at least) two different transport systems involved in Rf uptake into/FAD export out of mitochondria. Moreover, they imply the existence of an FAD synthetase activity inside mitochondria.

To prove the existence of FAD synthetase in SCM, solubilized mitochondria were incubated with or without the substrates of FAD synthetase, i.e. FMN and ATP, and the FAD synthesis rate was measured enzymatically as in (4, 11). A typical experiment is shown in Fig. 2. When solubilized SCM were incubated in the absence of substrates, a decrease of NADH absorbance was observed. Its rate increased with time up to a constant value (0.004 ΔA x min^{-1} after 6 min). This value corresponded to

FIG. 2

approximately 100 pmol x (mg protein)$^{-1}$ mitochondrial endogenous FAD, which is loosely and/or not bound to protein. When solubilized SCM were added with FMN and ATP for 2 min, the rate of the decrease in absorbance significantly increased (0.010 ΔA x min^{-1}). This value corresponded to about 400 pmol x (mg protein)$^{-1}$ of FAD. By subtracting the former FAD value from the latter, 300 pmol x (mg protein)$^{-1}$ of newly synthesized FAD were calculated as a result of 2 min incubation with the substrate pair of FAD synthetase. It should be noted that in the FAD synthetase assay, sodium pyrophosphate was present to prevent FMN dephosphorylation (Barile M.; unpublished result), which competes with FAD synthesis and which is possibly the result of an FMN phosphohydrolase previously observed in SCM and rat liver mitochondria (RLM) (10, 7). The amount of FAD synthesized in solubilized SCM starting from FMN and ATP increased with the incubation time, reaching a maximum at 5 min incubation. Incubation for longer time resulted in a progressive decrease of the newly synthesized FAD, probably due to FAD hydrolysis via FAD pyrophosphatase (7, 8). The rate of FAD synthesis, as measured in the interval of time in which the amount of newly synthesized FAD increased linearly with time, was found to be 150 ± 63 pmol x min^{-1} x (mg protein)$^{-1}$, in three experiments carried out with different mitochondrial preparations.

To exclude any contamination from cytosolic proteins, FAD synthesis was assayed in solubilized mitochondria and spheroplasts. FAD synthetase distribution was compared with those of CS and PDC, mitochondrial and cytosolic marker enzymes, respectively. In a typical assay (see the histograms in Fig. 2), the solubilized SCM were proven to synthesize FAD with a specific activity of 85 pmol x min^{-1} x (mg protein)$^{-1}$. This value corresponded to about 37% of that measured in the spheroplasts. In the same experiment, the specific activity of PDC measured in the mitochondrial fraction was only 5% of that measured in the spheroplasts. The purity of the mitochondrial fraction was tested by measuring the enrichment of the CS specific activity, which was 4-fold higher than that measured in the spheroplasts.

These results demonstrate that besides the cytosolic FAD synthetase, encoded by *FAD1* (9), mitochondria also possess an enzyme that performs FAD synthesis from the Rf imported from the cytosol.

Involvement of the Flx1p carrier in FAD export out of mitochondria. Due to the proposed absence of FAD synthetase inside mitochondria it had been proposed previously that Flx1p be involved in the mitochondrial import of cytosolically synthesized FAD (10).

To re-evaluate the function of the Flx1p carrier in awareness of the presence of FAD synthetase in SCM, and to gain further insights into the mechanisms of flavin traffic across the mitochondrial membrane, Rf uptake experiments were repeated by using mitochondria isolated from an *flx1*Δ mutant strain (Fig. 3). As in the wild-type strain, Rf uptake was accompanied by intramitochondrial FAD synthesis, both in 0-2 μM (Fig. 3A) and in 0-30 μM Rf concentration ranges (Fig. 3B), with no significant change of FMN content. Differently from the wild-type strain, no FAD export could be detected in the supernatant of mitochondria from the *flx1Δ* mutant strain. The dependence of the apparent Rf uptake rate on extramitochondrial Rf concentration in mitochondria from *flx1Δ* mutant cells (Fig. 3A) showed again saturation characteristics, with pseudo K_m and V_{max} values equal to 0.9 μM and 27 pmol x min^{-1} x $(mg\ protein)^{-1}$, respectively. A sigmoidal shape curve fitted the data of Rf uptake obtained in the higher concentration interval (Fig. 3B), with pseudo $S_{0.5}$ and V_{max} values of the same extent of that found in the wild-type strain (20 μM and 11900 pmol x min^{-1} x mg $protein^{-1}$, respectively).

FIG. 3

In spite of the kinetical limitations, the results obtained with the *flx1Δ* mutant strain fit the same hypothesis made for the wild-type strain; i.e. (at least) two distinct uptake systems are involved in Rf transport into mitochondria. Moreover, FAD export into the extramitochondrial phase was proven to depend on the activity of a distinct export system, which presumably is the Flx1p carrier.

Consistently, it was ascertained that the ability of FAD export, abolished in the mitochondria of the *flx1Δ* mutant strain, was restored in the mitochondria of the *flx1Δ* mutant strain transformed with the *FLX1* gene on a multicopy plasmid (Fig. 4). A control was made that the inability to export FAD is specifically due to the *FLX1* deletion and that it is not an undesired consequence of the poor growth capacity of the cell on the glycerol-containing medium (see below). Therefore, the same experiments were performed with mitochondria isolated from the wild-type and the *flx1*Δ mutant cells grown on

FIG. 4

medium containing ethanol as the carbon source. No significant change in Rf uptake efficiency was found, whereas the FAD export ability was reduced up to 80% in the mitochondria from *flx1*Δ mutant cells (not shown).

The involvement of an export system for FAD, which is different from the Rf uptake systems, was further confirmed by studying the inhibitor sensitivity of the transport process. To this aim, the effect of lumiflavin, a Rf analogue able to inhibit plasma membrane transport of the vitamin (25, 27), was studied. Results from a typical experiment carried out with wild-type SCM are shown in Table I. Lumiflavin, which apparently permeates the mitochondrial membrane, was found to significantly reduce the rate of FAD export. At 0.8 µM Rf, the export of FAD was completely inhibited by 15 µM lumiflavin. At 1.5 µM riboflavin, the FAD export rate was inhibited up to 44%. Conversely, lumiflavin did not significantly change the apparent rate of Rf uptake. When the same experiment was repeated by using mitochondria isolated from *flx1Δ* mutant cells, no effect of lumiflavin was observed both on the apparent Rf uptake rate and on the FAD synthesis rate in the pellet. This result allows to exclude that lumiflavin inhibits either the riboflavin uptake system(s) or the intramitochondrial FAD synthetic machinery.

Table I

To prove that Flx1p is neither involved in flavin cofactor import nor strictly required in maintaining flavin cofactor homeostasis within the organelles, in other experiments SCM from wild-type and *flx1*Δ mutant strain were compared with respect to both the endogenous flavin content and the capability to take up FAD and FMN. No significant change in the endogenous flavin cofactor level was found in SCM when cells were grown on glycerol (Table II), or ethanol (not shown). On the other hand, a significant impairment of mitochondrial cofactor levels was found in *flx1*Δ mutant cells grown on galactose, in agreement with (10) (not shown). When SCM were incubated with FAD in the 0-2 µM concentration range, we could not detect any significant increase in flavin cofactor inside mitochondria. Thus, we raised the FAD concentration to 10 µM. Results in Table II show that the deletion of the *FLX1* gene does not change the capability of SCM to take up both FAD and FMN and to synthesize FAD from FMN taken up. As a control, parallel experiments were performed to prove that mitochondria from *flx1*Δ mutant cells transformed with *FLX1* gene on a multicopy plasmid do

Table II

not significantly differ neither for the endogenous flavin cofactor level, nor for the ability to take up FMN and FAD (not shown). These results confirm the proposal that the Flx1p carrier is responsible for FAD export into the extramitochondrial phase, and they exclude that it is required for FAD and FMN uptake into mitochondria.

Involvement of Flx1p in ensuring a correct level of FAD-binding enzymes in the mitochondrial matrix. As expected in cells suffering from a mitochondrial defect, *flx1*Δ mutant cells did not grow on agar plates containing glycerol or pyruvate as a carbon source. They could grow on glucose, as efficiently as the wild-type strain, and they grew a little bit slower on ethanol. Mutant cells grew quite efficiently in liquid rich medium containing ethanol, whereas on lactate, pyruvate or glycerol they grew considerably slower than the wild-type strain. The growth defects were restored by complementing the deletion strain with the YEpFLX1 plasmid (not shown).

The growth phenotype was not caused by a general impairment of mitochondrial bioenergetics. In fact, we excluded the possibility that the potential across the mitochondrial membrane was affected in the deletion strain, as both wild-type and *flx1Δ* mutant cells accumulated the fluorescent dye DASPMI in a similar manner. Moreover, we did not find any significant difference in the ability of isolated SCM to carry out an efficient oxidative phosphorylation, as assessed by polarographic measurements of the oxygen consumption rate starting from either NADH or ascorbate plus tetramethyl-p-phenylenediamine (TMPD) (data not shown).

It was previously demonstrated that Flx1p is involved in maintaining the activity of intramitochondrially located FAD-binding enzymes, LPDH and SDH (9, 10). Therefore, in a series of experiments the ability of freshly isolated mitochondria from *flx1*Δ mutant cells to respire pyruvate plus malate and succinate, via pyruvate dehydrogenase (PDH) and SDH, respectively, was tested. A significant reduction in the oxygen uptake rate induced by pyruvate plus malate was found especially when cells had been grown on glycerol. This reduction is possibly due to a reduction in PDH activity, since, under the experimental conditions used, the PDH catalyzed reaction is the rate-limiting step of the overall process of pyruvate respiration (not shown). Conversely, no significant change in the

ability to respire succinate was found. Using phenylsuccinate as an impermeable inhibitor of dicarboxylate carrier (28), we applied the control strength analysis, essentially as described in (29 and references herein). We concluded that SDH is not the rate-limiting step of respiration, under the experimental conditions used (not shown).

To investigate whether in *flx1*Δ mutant cells the function of mitochondrial FAD-binding enzymes is affected, the activities of LPDH (E_3 in PDH complex) and SDH were measured in solubilized mitochondria (Table III). The deletion of *FLX1* resulted in a significant decrease in LPDH activity (40-70% decrease compared to the wild-type strain). Interestingly, the change in SDH activity was found to depend on the carbon source. A significant reduction in SDH activity (ranging between 60 and 80 % compared to the wild-type strain) was found in mitochondria from the *flx1*Δ mutant cells grown on glycerol, whereas no significant change was found in SCM from the cells grown on ethanol.

Table III

To test whether the reduction in FAD-binding enzyme activity observed in *flx1*Δ mutant cells is correlated with a reduction in the amount of protein and/or with a reduction in its level of flavinylation, the amount of SDH (the sole covalently flavinylated protein in SCM) was determined by Western blotting and immunological detection with an antibody directed against the flavin moiety of the SDH-Fp and by means of an analysis of flavin fluorescence of protein bands on SDS-PAGE gels (Fig. 5). When analyzing SCM isolated from the glycerol-grown wild-type cells, the flavinylated SDH-Fp is detectable with an M_r of 67 kDa (Fig. 5A). A 70% decrease in the amount of flavinylated SDH-cross-reacting material was found in SCM from glycerol-grown *flx1*Δ mutant cells. The amount of the flavinylated protein was restored in mitochondria isolated from *flx1*Δ mutant cells complemented with the YEpFLX1 plasmid. In agreement with the enzymatic activity assays, no significant change in the amount of flavinylated SDH-Fp was found in mitochondria isolated from ethanol-grown cells. The specific degree of flavinylation of SDH-Fp was estimated in another experiment (Fig. 5B), in which the amount of the UV-fluorescence of the SDH-Fp band on the gel (corresponding to the SDH-Fp covalently linked with flavin cofactor) was correlated with the protein content of the same band, as estimated by densitometric analysis of the Coomassie Blue stained gel (26). A similar reduction in both SDH-Fp fluorescence and protein content was observed in

FIG. 5

mitochondria isolated from the *flx1*Δ mutant strain grown on glycerol, as compared to mitochondria isolated from the wild-type strain. Thus, the specific degree of SDH-Fp flavinylation remained unchanged in *flx1*Δ mutant cells, whereas the total amount of flavinylated SDH-Fp was decreased. Both the fluorescence and the protein content of the SDH-Fp band was restored in SCM isolated from *flx1*Δ mutant cells complemented with YEpFLX1 plasmid. In agreement with the enzymatic assay, no significant change in both the fluorescence and the protein content of the SDH-Fp band was found in SCM from ethanol-grown cells. Thus, the reduction in the amount of flavinylated active SDH in *flx1Δ* mutants is specific for the carbon source and it reflects a decrease in the amount of SDH-Fp protein, rather than a reduction in its flavinylation level. These results demonstrate that the growth phenotype of the *flx1Δ* mutants is due to the fact that mitochondrial FAD-binding enzymes are present at significant lower concentrations as compared to the wild-type cells.

DISCUSSION

The experiments described here show that yeast mitochondria can take up Rf and exhibit a functional FAD synthesis pathway. Rf uptake occurs via (at least) two carrier systems that operate at different concentration ranges and perform different uptake efficiencies. The lower concentration range of Rf used in the uptake experiments (Figs 1A, 3A), roughly corresponds to the physiological concentration of the vitamin measured in spheroplasts by HPLC (0.8-1.5 μM). The mechanisms by which the homeostasis of the vitamin outside the organelle is regulated remain to be fully understood. In the cytosol, Rf is supposed to be generated by *de novo* synthesis (30). Nevertheless, the contribution of organelle compartmentation (31) and/or the occurrence of a putative flavin recycling pathway (4) cannot be excluded. Moreover, we cannot exclude that under certain physiological conditions, the uptake system operating at a higher concentration of the vitamin (2-30 μM range) could become relevant. Further experiments are in progress to characterize the mitochondrial Rf uptake carrier system(s), in particular to identify a suitable inhibitor for Rf uptake. Unfortunately, neither the SH reagent mersalyl nor atractyloside, which were found to inhibit Rf uptake and FAD export in RLM (4), inhibit Rf uptake in isolated SCM.

Conversely, we could demonstrate that the structural Rf analogue lumiflavin specifically prevents Rf-induced FAD export. When lumiflavin was added, Rf uptake and its intramitochondrial metabolization occurred independently from FAD export. This allows concluding that FAD export into the extramitochondrial phase occurs via an export system, which is different from the Rf uptake system(s). Indeed, FAD export could also be prevented by deletion of the *FLX1* gene. FAD export presumably occurs via Flx1p, since *flx1*Δ mutant cells lost their ability to export FAD from the organelle, but they did not lose their ability to accumulate Rf and to synthesize FAD. Moreover, the ability to export FAD was restored in *flx1*Δ mutant cells transformed with the *FLX1* gene on a multicopy plasmid. Nevertheless, we cannot exclude alternative hypotheses to explain our results; i.e. Flx1p could be a regulator of an unknown FAD efflux carrier.

Whatever the molecular mechanism is, the scenario emerging from our experiments is that in yeast, as well as in rat liver (4), the main source of intramitochondrial flavin cofactors is cytosolic Rf, rather than FAD synthesized in the cytosol and imported via Flx1p. This proposal is in line with the observation that virtually all FAD in the cytosol is protein-bound, as determined by ultrafiltration of the cytosolic fraction followed by HPLC analysis of both the retentate and the protein-free filtrate, in agreement with (32). The observation that deletion of *FLX1* does not necessarily change the intramitochondrial flavin cofactor level is consistent with this proposal. This conclusion is also in line with the occurrence of FAD synthesis inside the organelle: in this paper we show by fractionation studies that SCM as well as RLM (4) contain their own specific FAD synthetase, therefore excluding that FAD synthesis is totally dependent on the cytosolic FAD synthetase, i.e. Fad1p (9). Mitochondrial FAD synthetase activity represents about 8% of the total activity detectable in spheroplasts, as estimated by comparing the distribution of the marker enzyme CS, and assuming that the highest amount of CS activity is present in the mitochondrial fraction (33). A similar distribution ratio was found for mitochondrial riboflavin kinase in yeast (12).

FAD synthesis inside mitochondria is expected to be a crucial event in regulating FAD-binding enzyme biogenesis. Indeed, a number of mitochondrial apo-flavoenzymes have been reported to be charged with their cofactors only inside the organelle (5, 6, 34, 35). The experiments described here

demonstrate that Flx1p plays a crucial role in maintaining a normal level of FAD-binding enzyme activity. Deletion of *FLX1* did not affect the level of flavin cofactors inside mitochondria or the degree of SDH-Fp flavinylation, but instead the amount of SDH-Fp protein. These observations disproved the previous proposal that the decrease in mitochondrial FAD-binding enzyme activities in *flx1*Δ mutant cells would be due to a reduced level of FAD cofactor inside mitochondria during holoenzyme biogenesis (9, 10). We could confirm that SDH activity is significantly reduced in *flx1*Δ mutant cells, but we then demonstrated that this is due to a strongly reduced amount of the SDH-Fp protein. We do not know whether this reduction is caused by an impairment of SDH synthesis or by destruction of the protein. Moreover, also LPDH activity was strongly reduced in *flx1*Δ mutant cells. In contrast to LPDH, the decrease in the amount of SDH could only be found in *flx1*Δ mutant cells grown on glycerol but not in those grown on ethanol. These observations might explain the growth phenotypes of the *flx1*Δ mutant cells: they did not grow on glycerol as a carbon source, but were able to grow on ethanol. In agreement with this, PDH is expected to be dispensable during growth on ethanol but not on glycerol (36) whereas SDH activity is necessary for growth on both kinds of carbon sources.

What is the role of Flx1p in the regulation of the amount of SDH-Fp protein? As we could not measure any effects of its deletion on flavin cofactor levels inside mitochondria it is tempting to speculate that Flx1 itself plays a more direct regulatory role. One possibility is that mitochondrial flavins while moving towards the cytoplasm via Flx1p have an additional signaling function besides acting as "classical" cofactors. Mitochondrial FAD (or a derivative) exported into the cytoplasm could become a messenger during FAD-binding enzyme biogenesis or degradation in the cytoplasm. Regulatory effects have already been described for Rf and thiamin derivatives in bacteria (37, 38). Another possibility would be that like other *S. cerevisiae* permeases (39-41), Flx1p *per se* might function as a sensor of vitamin/cofactor availability. In this respect, it could use mitochondrial signaling molecules or detect the flow of FAD into the cytosol to allow cross-talk between the flavin content and metabolic needs of the mitochondria and the synthesis or turn-over machinery of the FAD-binding enzymes in the cytosol.

Acknowledgements

The authors wish to thank Dr. T. Rinaldi, Dipartimento di Biologia Cellulare e dello Sviluppo, Università di Roma "La Sapienza", for performing fluorescence microscopy. The helpful cooperation of Dr. C. De Virgilio, who took part in the initial stages of this work, and the excellent technical assistance of Mr. V. Giannoccaro, are gratefully acknowledged. This work was supported by grants from Telethon (Grant n. 1205) and the University of Bari (Fondi di Ateneo per la Ricerca) to M. B. V. B. was supported by fellowship from ESF (P.O.P. 1994-1999). T. A. G. and D. B. were supported by fellowship from ESF (P.O.P. 2000-2006). C. B. was a recipient of a post-graduate research fellowship (Assegno di Ricerca) financed by Telethon and by the University of Bari.

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FIGURE LEGENDS

Fig. 1. Rf uptake and FAD export from SCM isolated from wild-type cells. SCM were incubated under the experimental conditions described in Experimental Procedures. The uptake reaction was started by the addition of Rf at the indicated concentrations and stopped 20 s later by rapid centrifugation. Rf actually taken up (◆), FAD in the mitochondrial pellet (❍) and in the supernatant (●, SN) were determined in neutralized extracts by HPLC as described in the Experimental Procedures. The *y*-axis represents the flavin transport/synthesis rates expressed as pmol x min^{-1} x mg $protein^{-1}$. Values are the mean of three replicates (± SE) performed using the same mitochondrial preparation.

Fig. 2. FAD synthesis as catalyzed by solubilized SCM. FAD amount was enzymatically assayed either in solubilized SCM or in solubilized SCM with added FMN and ATP, as described in Experimental Procedures. In the inset, the specific activities of FAD synthetase measured in spheroplasts and SCM are compared with those of PDC and CS.

Fig. 3. Rf uptake and FAD synthesis in SCM isolated from *flx1Δ* mutant cells. SCM isolated from *flx1Δ* mutant cells were incubated with Rf under the same experimental conditions as described in Fig. 1. Rf actually taken up (◆), FAD in the mitochondrial pellet (❍) and in the supernatant (●, SN), were determined as described in the Experimental Procedures. The *y*-axis represents the flavin transport/synthesis rates expressed as pmol x min^{-1} x mg $protein^{-1}$. Values are the mean of three replicates (± SE) performed using the same mitochondrial preparation.

Fig. 4. FAD export from SCM isolated from wild-type, *flx1Δ* mutant and *flx1Δ* mutant cells transformed with YEpFLX1 plasmid. SCM were incubated under the experimental conditions described in Experimental Procedures. The uptake reaction was started by the addition of Rf and stopped 1 min later by rapid centrifugation. The amount of FAD exported was determined in neutralized extracts of the supernatants by HPLC. Values are the mean of three replicates (± SE) performed using the same mitochondrial preparation.

Fig. 5. Detection of flavinylated SDH-Fp. Wild-type, *flx1Δ* mutant and *flx1Δ* mutant cells transformed with YEpFLX1 plasmid, were grown on glycerol or ethanol as carbon source. Proteins from SCM were separated by SDS-PAGE and analyzed as described under Experimental Procedures. **A) Western blotting.** Covalently flavinylated SDH-Fp (FAD-SDH-Fp) was detected with anti-FAD specific antibodies and its amount densitometrically evaluated (a). **B) Flavin fluorescence detection.** The flavin fluorescence of SDH-Fp was visualized by UV irradiation of the unstained gel. In (b), the SDH-Fp fluorescence intensity was evaluated (black bars) and compared with the amount of SDH-Fp protein revealed by Coomassie staining (white bars).

TABLE I

Inhibition by lumiflavin of FAD export from SCM isolated from wild-type cells. SCM were incubated for 1 min in the presence or absence of lumiflavin (15 µM), as described in Experimental Procedures. The uptake reaction was started by the addition of riboflavin at the indicated concentrations and stopped 20 s later by rapid centrifugation. Riboflavin taken up in the mitochondrial pellet and FAD exported in the supernatants were determined in neutralized extracts by HPLC. Flavin transport rates are expressed as pmol x min^{-1} x mg $protein^{-1}$. Values are the mean of three replicates (± SE) performed using the same mitochondrial preparation.

	Flavin transport rate (pmol x min^{-1} x mg $protein^{-1}$)	
Addition	Riboflavin uptake	FAD export
Riboflavin (0.8µM)	4 ± 4	119 ± 21
Riboflavin (0.8µM) + Lumiflavin	8 ± 3	0[a]
Riboflavin (1.5µM)	16 ± 6	254 ± 12
Riboflavin (1.5µM) + Lumiflavin	28 ± 4	140 ± 30

[a]0, not detectable

TABLE II

FAD and FMN uptake in SCM isolated from wild-type and *flx1*Δ mutant cells. SCM were incubated under the experimental conditions described in Experimental Procedures. The uptake reaction was started by the addition of either FAD or FMN (10 µM each) and stopped 1 min later by rapid centrifugation. Flavin content in mitochondrial pellets was determined by HPLC. Values are given as the mean (± SE) of three experiments (each carried out in three replicates) performed with different mitochondrial preparations.

		Mitochondrial flavin content (pmol x mg $protein^{-1}$)		
Strain	Addition	FAD	FMN	riboflavin
wild-type	none	160 ± 10	30 ± 10	0[a]
	FAD	398 ± 123	57 ± 28	5 ± 3
	FMN	208 ± 55	109 ± 18	32 ± 19
flx1Δ	none	140 ± 30	40 ± 10	0
	FAD	476 ± 124	57 ± 21	5 ± 1
	FMN	201 ± 43	152 ± 18	33 ± 7

[a]0, not detectable

TABLE III

Lipoamide dehydrogenase and succinate dehydrogenase activities in SCM isolated from wild-type and *flx1Δ* mutant cells. SCM were isolated from cells grown on either glycerol- or ethanol-containing medium and mitochondrial enzyme specific activities were measured as reported in Experimental Procedures. Values are given as the mean (± SE) of three experiments performed with different mitochondrial preparations. Statistical evaluation was carried out according to the Student's *t*-test.

		Specific activity (μmol x min^{-1} x mg $protein^{-1}$)	
Carbon source	Strain	Lipoamide dehydrogenase	Succinate dehydrogenase
Glycerol	wild-type	0.042 ± 0.011	0.127 ± 0.011
	flx1Δ	0.021 ± 0.007[a]	0.048 ± 0.001[a]
Ethanol	wild-type	0.094 ± 0.032	0.159 ± 0.045
	flx1Δ	0.026 ± 0.008[a]	0.154 ± 0.008

[a] Values significantly different from that measured in wild-type SCM, $P < 0.05$.

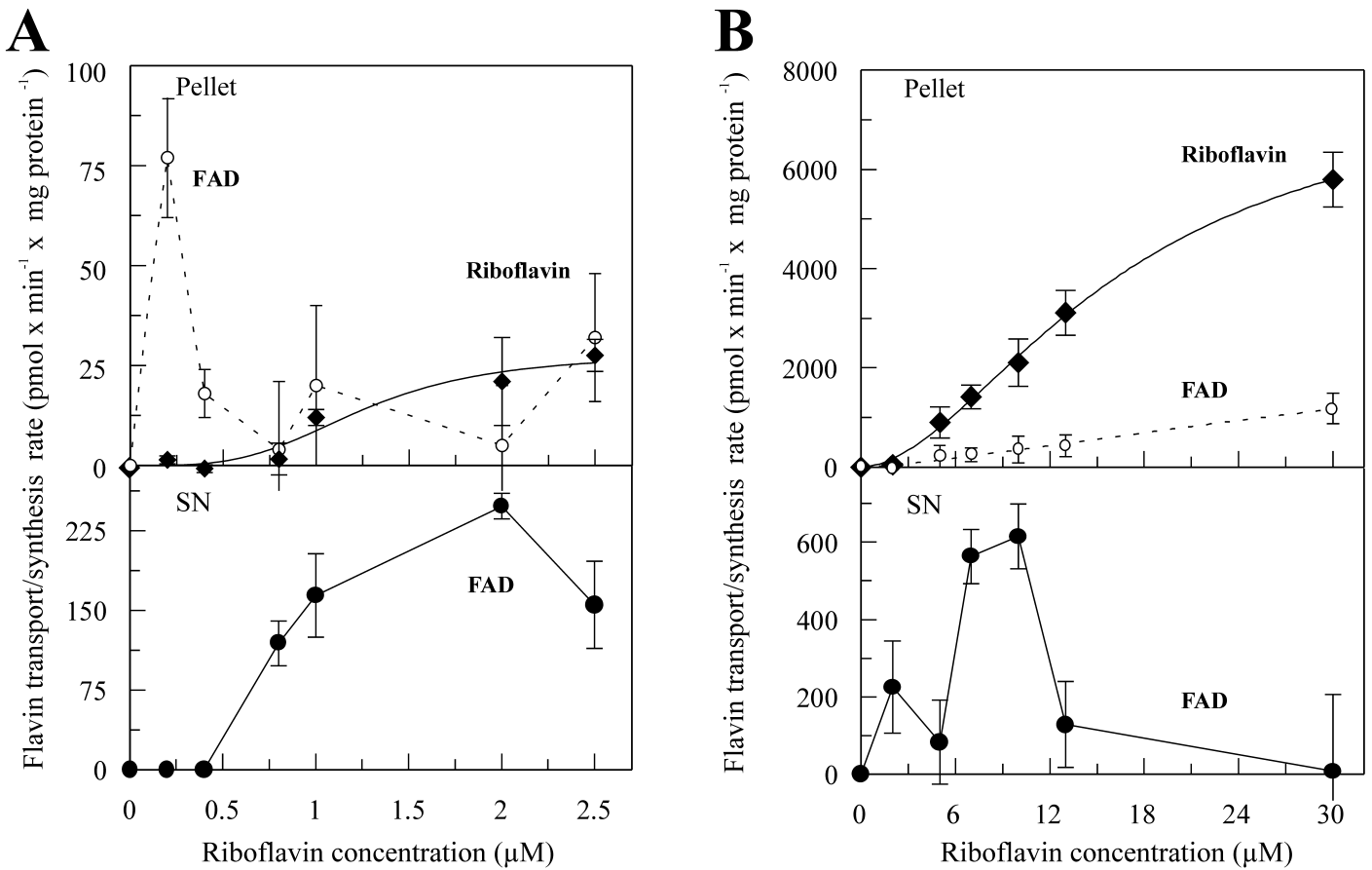

A
B
Pellet
FAD
Riboflavin
SN
FAD
Flavin transport/synthesis rate (pmol x min-1 x mg protein-1)
Riboflavin concentration (µM)
0
0.5
1
1.5
2
2.5
100
75
50
25
225
150
6
12
18
24
30
8000
6000
4000
2000
600
400
200

Figure 1

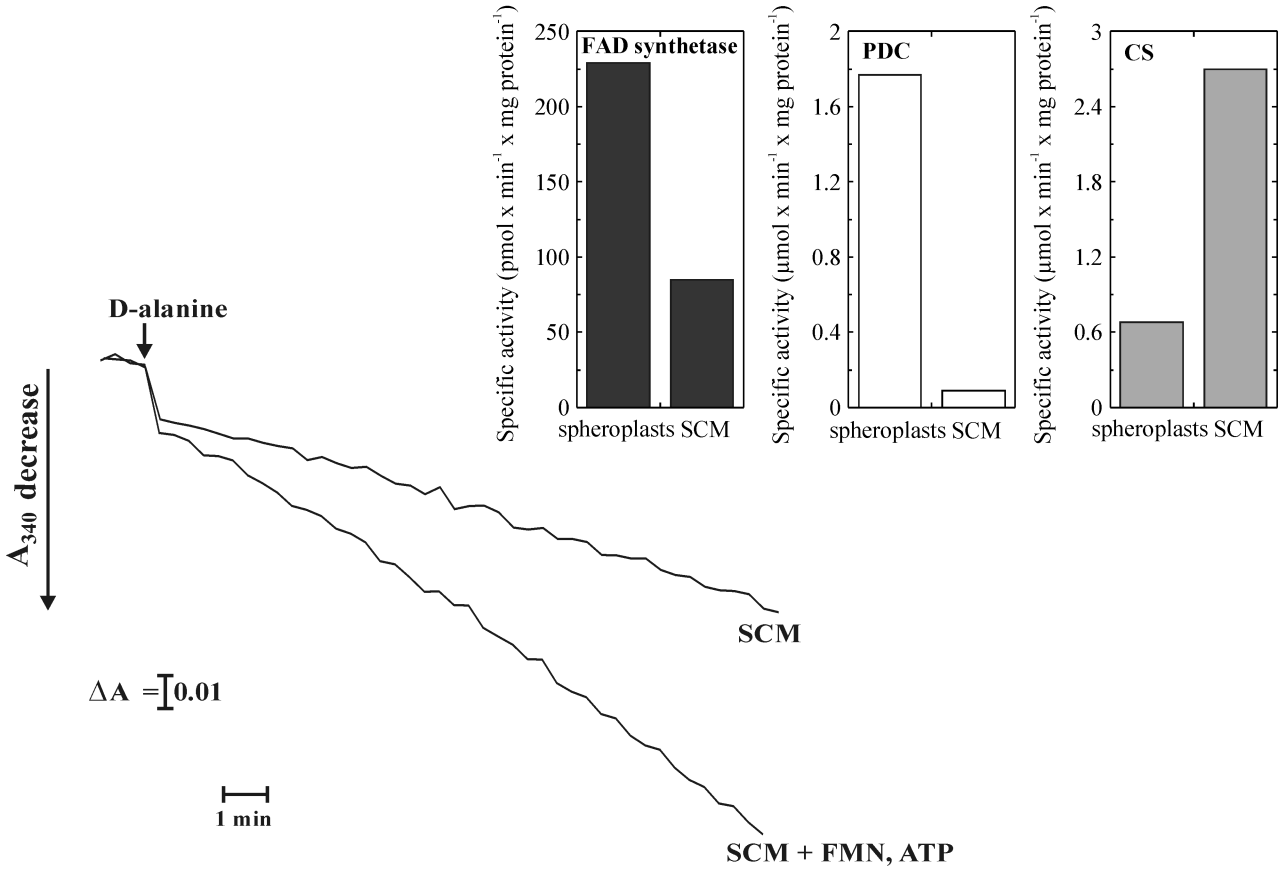

FAD synthetase
Specific activity (pmol x min-1 x mg protein-1)
250
200
150
100
50
0
spheroplasts SCM
PDC
Specific activity (µmol x min-1 x mg protein-1)
2
1.6
1.2
0.8
0.4
0
spheroplasts SCM
CS
Specific activity (µmol x min-1 x mg protein-1)
3
2.4
1.8
1.2
0.6
0
spheroplasts SCM
D-alanine
A340 decrease
SCM
ΔA = 0.01
1 min
SCM + FMN, ATP

Figure 2

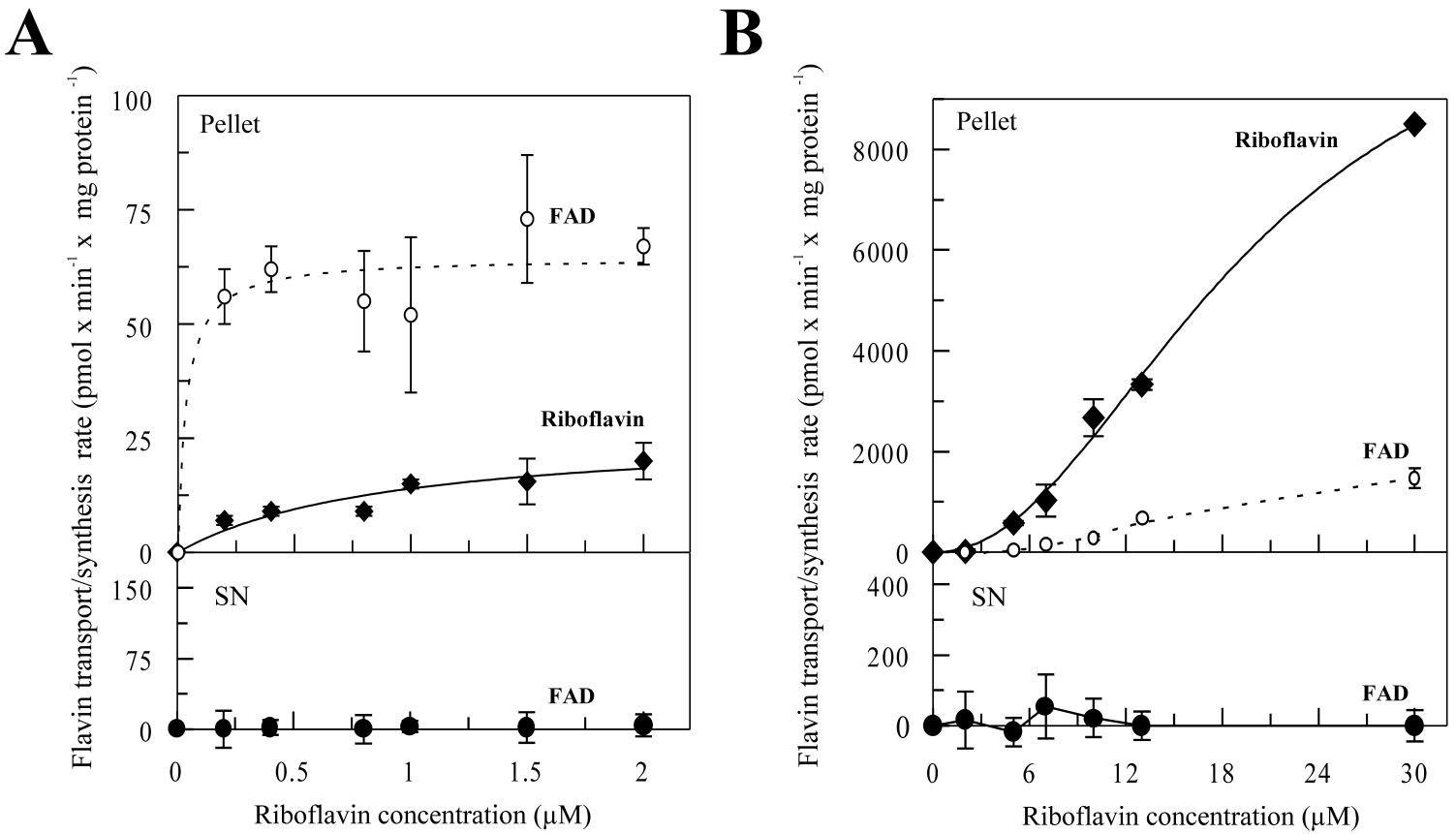

A
B
Pellet
SN
FAD
Riboflavin
Flavin transport/synthesis rate (pmol x min-1 x mg protein-1)
Riboflavin concentration (µM)
0
0.5
1
1.5
2
6
12
18
24
30
25
50
75
100
150
200
400
2000
4000
6000
8000

Figure 3

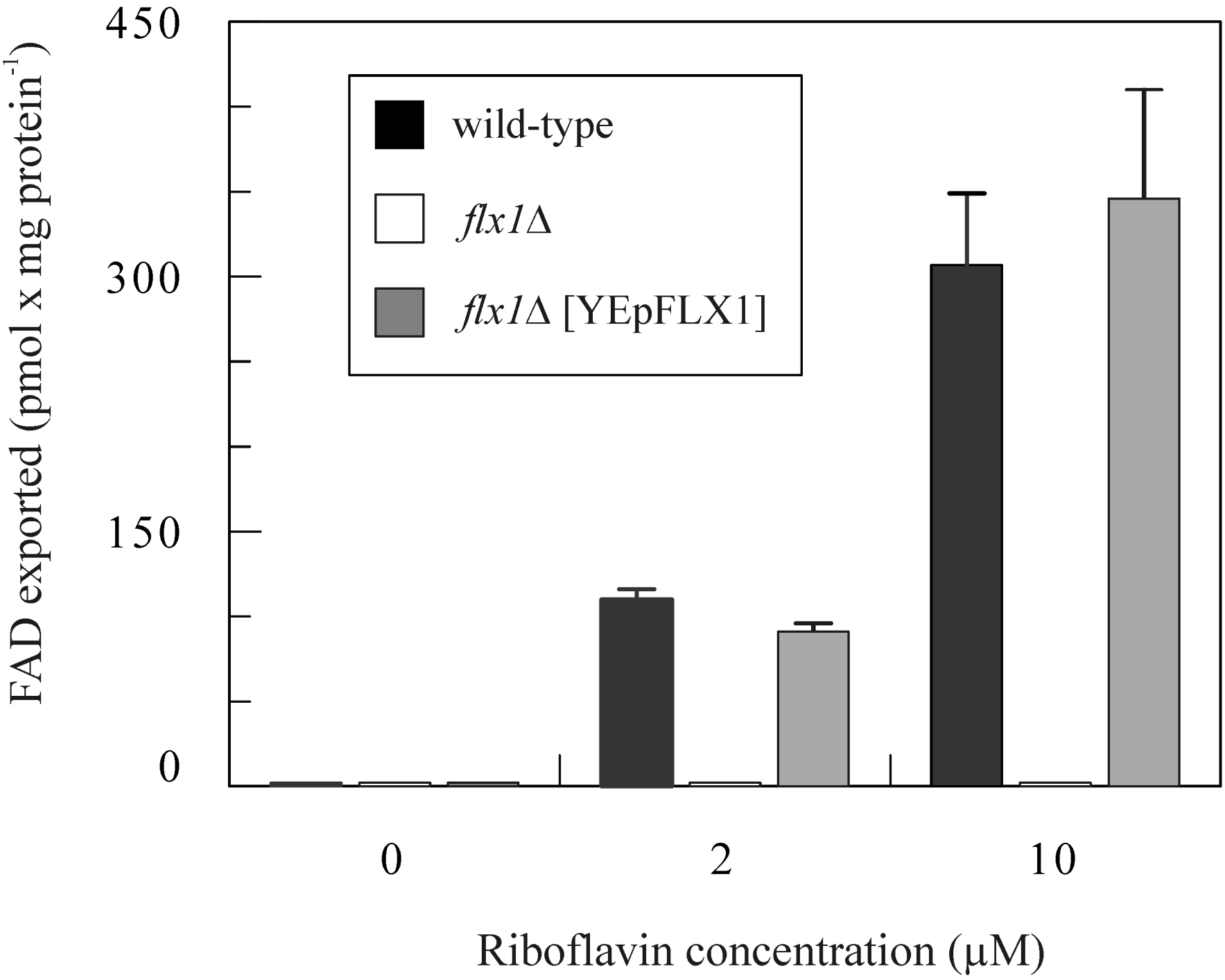
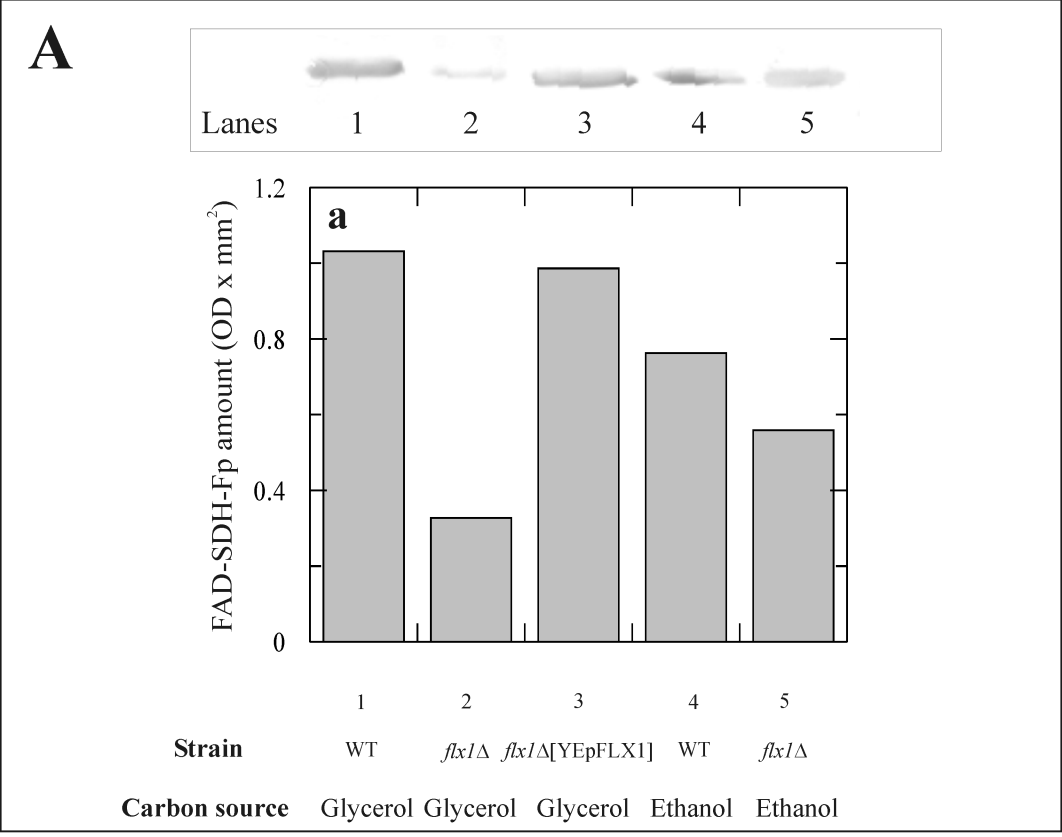

450
300
150
0
FAD exported (pmol x mg protein-1)
wild-type
flx1Δ
flx1Δ [YEpFLX1]
0
2
10
Riboflavin concentration (μM)

Figure 4


A
Lanes 1 2 3 4 5
a
FAD-SDH-Fp amount (OD x mm2)
1.2
0.8
0.4
0
1 2 3 4 5
Strain WT flx1Δ flx1Δ[YEpFLX1] WT flx1Δ
Carbon source Glycerol Glycerol Glycerol Ethanol Ethanol

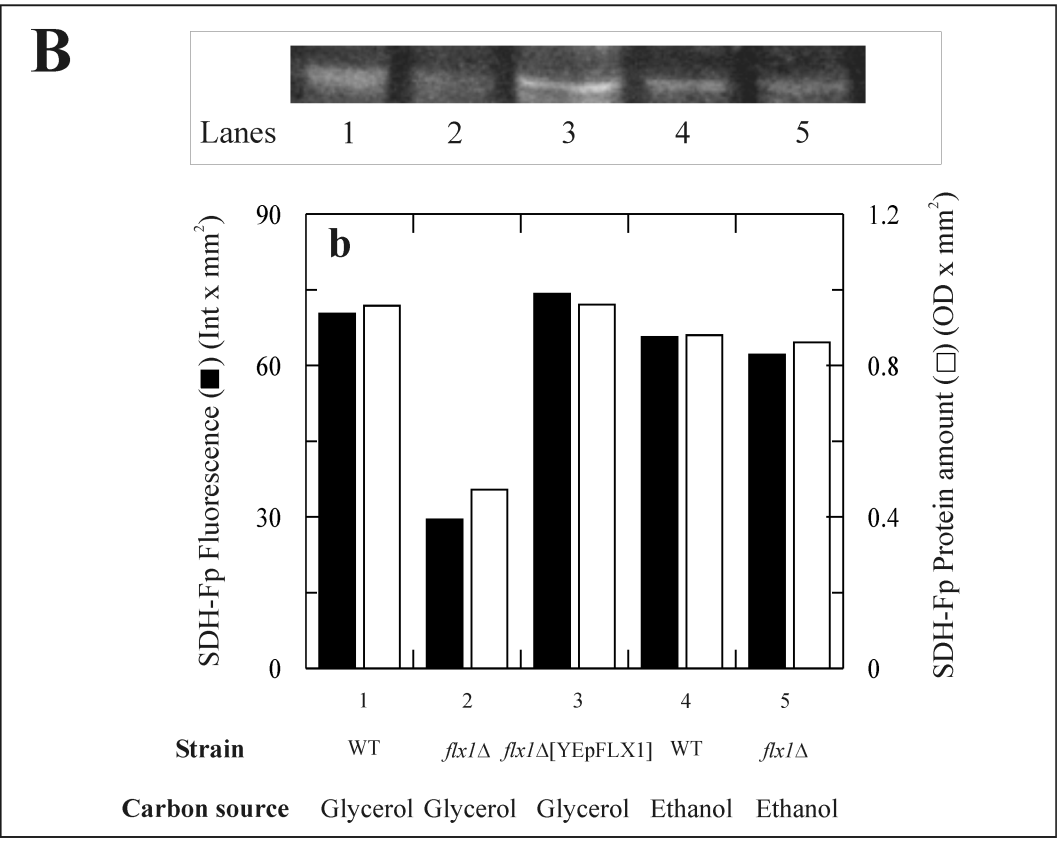

B
Lanes 1 2 3 4 5
b
SDH-Fp Fluorescence (■) (Int x mm2)
SDH-Fp Protein amount (□) (OD x mm2)
90
60
30
0
1.2
0.8
0.4
0
1 2 3 4 5
Strain WT flx1Δ flx1Δ[YEpFLX1] WT flx1Δ
Carbon source Glycerol Glycerol Glycerol Ethanol Ethanol

Figure 5

Riboflavin uptake and FAD synthesis in Saccharomyces cerevisiae mitochondria. Involvement of the Flx1p carrier in FAD export

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J. Biol. Chem. published online October 10, 2003
