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The occurrence of riboflavin kinase and FAD synthetase ensures FAD synthesis in tobacco mitochondria and maintenance of cellular redox status

Teresa A. Giancaspero[1], Vittoria Locato[2], Maria C. de Pinto[3], Laura De Gara[2,3] and Maria Barile[1]

1 Dipartimento di Biochimica e Biologia Molecolare 'E. Quagliariello', Università degli Studi di Bari, Italy
2 Centro Interdipartimentale di Ricerche Biomediche (CIR), Università Campus Biomedico, Roma, Italy
3 Dipartimento di Biologia e Patologia Vegetale, Università degli Studi di Bari, Italy

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Correspondence
M. Barile, Dipartimento di Biochimica e Biologia Molecolare 'Quagliariello' Università degli Studi di Bari, Via Orabona 4, I-70126 Bari, Italy
Fax: +39 0805443317
Tel: +39 0805443604
E-mail: m.barile@biologia.uniba.it

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Intact mitochondria isolated from *Nicotiana tabacum* cv. Bright Yellow 2 (TBY-2) cells can take up riboflavin via carrier-mediated systems that operate at different concentration ranges and have different uptake efficiencies. Once inside mitochondria, riboflavin is converted into catalytically active cofactors, FMN and FAD, due to the existence of a mitochondrial riboflavin kinase (EC 2.7.1.26) and an FAD synthetase (EC 2.7.7.2). Newly synthesized FAD can be exported from intact mitochondria via a putative FAD exporter. The dependence of FMN synthesis rate on riboflavin concentration shows saturation kinetics with a sigmoidal shape ($S_{0.5}$, V_{max} and Hill coefficient values 0.32 ± 0.12 μM, 1.4 nmol·min^{-1}·mg^{-1} protein and 3.1, respectively). The FAD-forming enzymes are both activated by $MgCl_2$, and reside in two distinct monofunctional enzymes, which can be physically separated in mitochondrial soluble and membrane-enriched fractions, respectively.

Whereas mammals must obtain riboflavin (Rf, vitamin B_2) from food, plants, along with fungi and bacteria, can synthesize Rf *de novo*. The primary role of Rf in cell metabolism derives from its conversion into FMN and FAD, the redox cofactors of a large number of dehydrogenases, reductases and oxidases [1].

Most flavoenzymes are compartmented in the cellular organelles, where they ensure the functionality of mitochondrial electron transport, photosynthesis, metabolism of fatty acids, some amino acids, choline and betaine, and synthesis of vitamin B_6, vitamin B_{12}, folate, and protoporphyrin. FAD is also the coenzyme of glutathione reductase, which mediates regeneration of reduced glutathione (GSH), a scavenger of free radicals and reactive oxygen species and a modulator of protein function by S-glutathionylation [2]. Ero1p- and sulfhydryl oxidase-dependent folding of secretory proteins also depend on FAD [3–5].

In plants, FAD is involved in ascorbate (ASC) biosynthesis and recycling, thus playing a crucial role in cell defence against oxidative stress and in programmed cell death [6–10]. Interestingly, the last enzyme in the ASC biosynthetic pathway, L-galactonolactone dehydrogenase (EC 1.3.2.3), is a mitochondrial flavoenzyme [11–15]. A mitochondrial isoform exists for all the other flavoenzymes involved in the ASC–GSH cycle [14]. Thus, we expect that in plants, as already demonstrated for human cells [2,16], Rf

Abbreviations
ADH, alcohol dehydrogenase; ASC, ascorbate; AtFMN/FHy, bifunctional riboflavin kinase FMN hydrolase; Cnp60p, mitochondrial chaperone 60; D-AAO, D-amino acid oxidase; EGFP, enhanced green fluorescent protein; FADS, FAD synthetase; FUM, fumarase; GSH, glutathione; M_{fr}, mitochondrial membrane-enriched fraction mt, mitochondria; PGI, phosphoglucoisomerase; RCI, respiratory control index; Rf, riboflavin; RK, riboflavin kinase; SDH, succinate dehydrogenase; SDH-Fp, succinate dehydrogenase flavoprotein subunit; S_{fr}, mitochondrial soluble fraction; TBY-2, *Nicotiana tabacum* cv. Bright Yellow 2.

deficiency or defective conversion of Rf into FAD might cause impairment of cellular redox status regulation. In plants, Rf treatment is also able to activate signal transduction pathways, thus conferring resistance to fungal infections [17]. This is in line with the additional regulatory roles of this vitamin, already described in yeasts [18], human cell lines [2] and patients suffering from Rf-responsive multiple acyl-CoA dehydrogenase deficiency [19].

Rf biosynthesis in plants, which has been described in some detail in the last decade, is nearly identical to that in yeast and bacteria. All of the enzymes of Rf biosynthesis identified to date seem to reside in plastids [17].

Conversion of Rf to FAD requires the sequential actions of riboflavin kinase [ATP:riboflavin 5′-phosphotransferase (RK); EC 2.7.1.26] and FAD synthetase [FMN:ATP adenylyltransferase (FADS); EC 2.7.7.2]. In yeasts, humans and rats, distinct monofunctional enzymes exist with either RK or FADS activity [20–24]. The corresponding genes have been identified and cloned for the first time in *Saccharomyces cerevisiae* [25,26] and more recently in humans [27,28]. In both rat liver and *S. cerevisiae*, FAD synthesis also occurs in mitochondria, by virtue of the existence of mitochondrial RK and FADS [26,29–32]. However, in prokaryotes, bifunctional enzymes with RK and FADS activity [33–35] and monofunctional enzymes with only RK activity [36] have been described. No monofunctional FAD synthetases have yet been found.

In plants, RK or FADS activity has been assayed previously [37–40], and a monofunctional RK was purified from mung bean [40]. In these earlier studies, subcellular localization of RK and FADS was not addressed, except for a single study carried out in spinach, which revealed RK activity in the cytosol and in an organellar fraction containing chloroplasts and mitochondria [41].

Recently, a bifunctional RK-FMN hydrolase (At-FMN/FHy), unique to plants, has been cloned and characterized [42]. The bioinformatic prediction of its localization is cytosolic. The cloning, recombinant expression and purification of two new monofunctional FADS enzymes from *Arabidopsis thaliana* (AtRibF1 and AtRibF2) was achieved by Sandoval *et al.* [43], as this article was being written. Both enzymes reside in plastids. Natural FADS activity was not detectable in Percoll-isolated chloroplasts from pea (*Pisum sativum*) [43]. As far as mitochondria are concerned, RK – but not FADS – activity was revealed in solubilized pea mitochondria [43]. The origin of mitochondrial FAD in plants still needs to be clarified.

Rf uptake and metabolism in intact coupled *Nicotiana tabacum* cv. Bright Yellow 2 (TBY-2) mitochondria have been studied to elucidate the mechanism by which plant mitochondria can provide their own FAD. The activities of RK and FADS were also determined in solubilized organelles. Our results are the first experimental evidence that TBY-2 mitochondria are able to take up Rf, to synthesize FAD, and to export FAD outside mitochondria.

Results

Rf uptake and FAD export by intact TBY-2 mitochondria

The experiments described here were aimed at ascertaining whether and how TBY-2 mitochondria are permeable to externally added Rf and whether Rf taken up can be processed to give the enzymatically active intramitochondrial cofactors FMN and FAD.

First, the purity of mitochondrial preparations starting from protoplasts, prepared as in [13], was assessed by following the enrichment of the membrane marker succinate dehydrogenase flavoprotein subunit (SDH-Fp) or of the matrix marker fumarase (FUM). As shown in Fig. 1, both proteins were about 15-fold enriched in the mitochondrial fraction and depleted in the fraction corresponding to plastids. The specific activities of plastid marker enzymes phosphoglucoisomerase (PGI, Fig. 1) and glutamate synthase (data not shown) were six-fold enriched in the plastid fraction and depleted in the mitochondrial fraction. The cytosolic marker enzyme alcohol dehydrogenase (ADH) [44] was significantly depleted, with a specific activity five-fold lower in the mitochondrial fraction than in protoplasts (Fig. 1).

The mitochondrial and the extramitochondrial amounts of Rf, FMN and FAD in the acid-extractable fractions were measured via HPLC and compared to the amounts of flavin cofactors in whole protoplasts and plastids (Table 1). In three experiments performed with different preparations, the endogenous FAD, FMN and Rf contents in TBY-2 mitochondria were equal to 290 ± 66, 132 ± 51 and 2 ± 1 pmol·mg^{-1} protein, respectively (Table 1). No flavin cofactor was detected in the postmitochondrial supernatant; this is in line with the mitochondrial membrane integrity. It should also be noted that plastids contain a significant amount of flavin cofactors, which tallies with the presence of the large number of flavoenzymes in this subcellular compartment [17].

As Rf metabolism is expected to depend on the organelle energy state, the functional features of

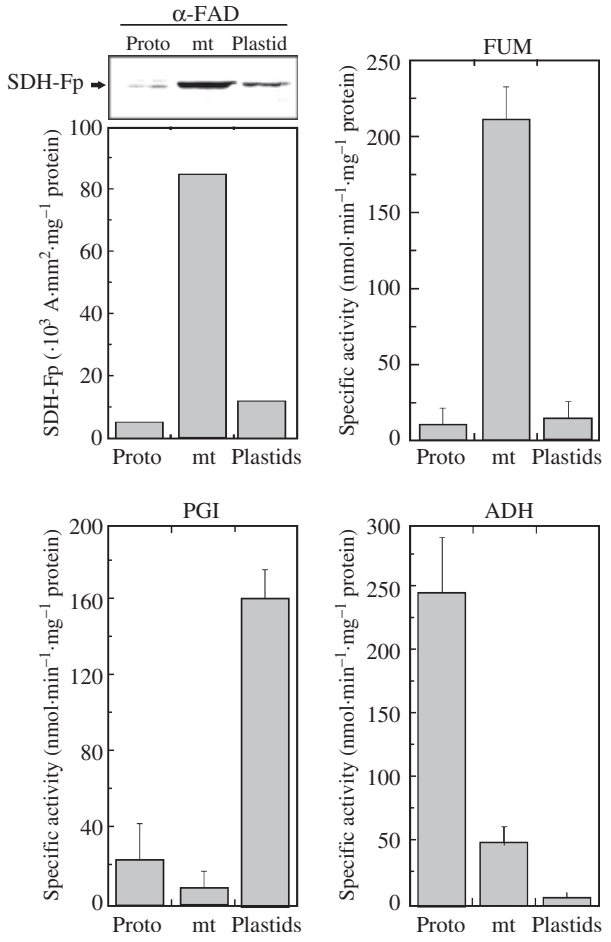

Fig. 1. Purity of TBY-2 mitochondria. In TBY-2 protoplasts (proto), mitochondria (mt) and plastids (0.05–0.1 mg) the amount of SDH-Fp, detected with α-FAD, and the FUM, PGI and ADH activities were measured, as reported in Experimental procedures. The values of the enzymatic activities are the mean (± SD) of three experiments performed with different cellular preparations.

TBY-2 mitochondria were checked in a series of preliminary experiments by polarographic measurements of the oxygen uptake rate starting from either NADH or succinate, essentially as in [18]. In a typical experiment (Fig. 2A), TBY-2 mitochondria respired with NADH (1 mM) at a rate equal to 61 nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ protein. When ADP (0.1 mM) was added, the oxygen uptake rate increased up to 164 nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ protein, with a respiratory control index (RCI) value equal to 2.7. When succinate (5 mM) was used as substrate (Fig. 2B), the oxygen uptake rate, equal to 47 nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ protein in the absence of ADP, increased up to 70 nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ protein in the presence of ADP (with an RCI value equal to 1.5). In three experiments, performed with different mitochondrial preparations, TBY-2 mitochondria showed RCI values ranging from 2.0 to 3.0 and from 1.4 to 1.8 with NADH and succinate, respectively, used as substrates.

Table 1. Endogenous flavin content in TBY-2 mitochondria. Intact TBY-2 mitochondria, resuspended in isotonic medium, were rapidly centrifuged at 15 000 ***g*** for 5 min to obtain a mitochondrial pellet and a postmitochondrial supernatant. Flavin content was determined in neutralized perchloric acid extracts of mitochondrial pellet, postmitochondrial supernatant, protoplasts and plastids by HPLC, as described in Experimental procedures. The means (± SD) of the flavin endogenous content determined in three experiments performed with different preparations are reported. ND, not detectable.

	Endogenous flavin content ($pmol \cdot mg^{-1}$ protein)		
	FAD	FMN	Rf
Mitochondrial pellet	290 ± 66	132 ± 51	2 ± 1
Postmitochondrial supernatant	ND	ND	ND
Protoplasts	246 ± 4	114 ± 2	10 ± 1
Plastids	842 ± 13	360 ± 10	13 ± 1

In a set of experiments, Rf (0.2–30 μM) was added to purified intact TBY-2 mitochondria, and flavin changes over the endogenous values were measured by HPLC. Experimental data were collected within the initial linear range of Rf uptake rates (i.e. 20 s of incubation) and were corrected for adherent/bound vitamin as described in Experimental procedures. Data were expressed as rates of flavin transport/synthesis in relation to Rf concentration (Fig. 3).

At the lower concentrations of Rf used (0.2–3.0 μM), no increase in mitochondrial Rf, FMN and FAD contents was observed (Fig. 3, mt Pellet), whereas FAD appeared in the extramitochondrial phase (Fig. 3, mt SN). This observation is consistent with the occurrence of FAD export into the postmitochondrial supernatant, following Rf import and intramitochondrial FAD synthesis. No appearance of FMN was observed in the postmitochondrial supernatant. Owing to the rapid conversion of Rf into FAD and its rapid efflux in the postmitochondrial supernatant, the rate of FAD export matched with the rate of Rf uptake (Fig. 3, mt SN). The dependence of the 'apparent' Rf uptake rate on vitamin concentration showed saturation characteristics, with a maximum of about 117 $pmol \cdot min^{-1} \cdot mg^{-1}$ protein at 0.4 μM (Fig. 3, mt SN). At Rf concentrations higher than 0.4 μM (Fig. 3, mt SN) the rate of FAD export decreased. These limitations prevented a detailed characterization of the transport process. However, by fitting the first set of data (up to 0.4 μM Rf) according to the Michaelis–Menten equation [Eqn (1) in Experimental procedures], 'apparent' K_m

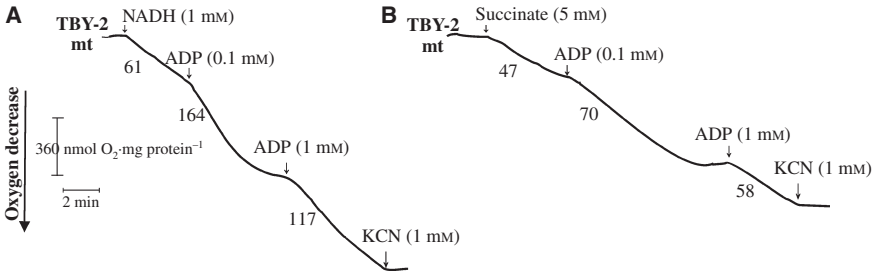

Fig. 2. Polarographic measurements of the NADH-dependent (A) and succinate-dependent (B) oxygen uptake rate in TBY-2 mitochondria. TBY-2 mitochondria (0.1 mg) were incubated in respiration medium, as described in Experimental procedures. The additions were made at the points indicated by arrows. The numbers along the trace refer to the oxygen uptake rate expressed as nmol O_2·min^{-1}·mg^{-1} protein.

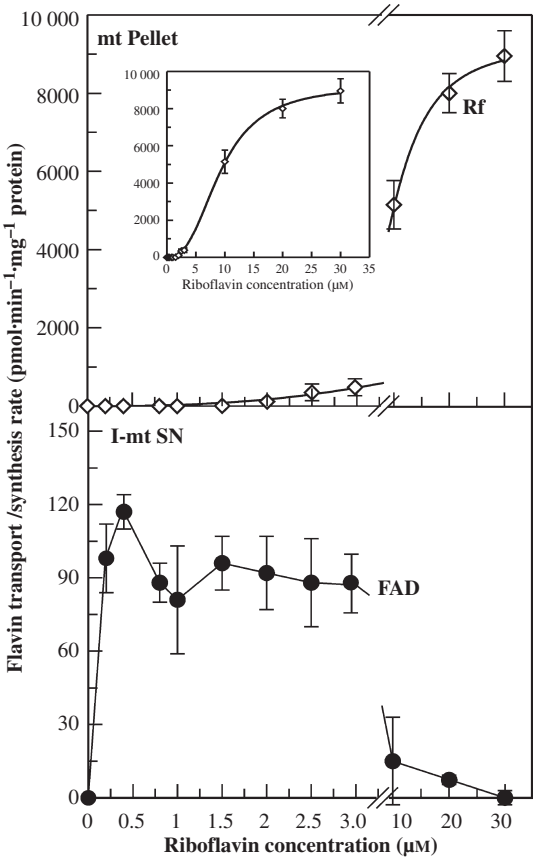

Fig. 3. Riboflavin uptake by and FAD export from intact TBY-2 mitochondria. Intact TBY-2 mitochondria (0.1–0.2 mg) were incubated at 2 °C in 500 μL of transport medium. The uptake reaction was started by addition of Rf at the indicated concentrations, and stopped 20 s later by rapid centrifugation. Rf actually taken up in the mitochondrial pellet (mt pellet) (◇) and FAD in the intact mitochondria supernatant (I-mt SN) (●) were determined in neutralized perchloric acid extracts by HPLC, as described in Experimental procedures. The *y*-axis represents the flavin transport/synthesis rates expressed as pmol·min^{-1}·mg^{-1} protein. Values are the mean of three replicates (± SD) performed using the same mitochondrial preparation.

and V_{max} were calculated; their values were 0.09 μM and 145 pmol·min^{-1}·mg^{-1} protein, respectively.

When Rf concentration was increased in the range from 10 to 30 μM, a significant increase in Rf amount was observed in the mitochondrial pellet (Fig. 3, mt Pellet), with a concomitant reduction in the rate of FAD appearance in the postmitochondrial supernatant (Fig. 3, mt SN). Under these experimental conditions, the dependence of the Rf uptake rate on the postmitochondrial supernatant showed saturation characteristics with a sigmoidal shape (Fig. 3, inset, mt Pellet). Data fitting was performed according to allosteric kinetics [Eqn (2) in Experimental procedures] with a Hill coefficient equal to 2.6. The kinetic parameters, expressed as 'pseudo' $S_{0.5}$ and V_{max}, were 9.2 μM and 9.3 nmol·min^{-1}·mg^{-1} protein, respectively.

To ensure that the FAD appearance observed at low Rf concentrations was not due to extramitochondrial metabolism, FMN (1 μM) and ATP (1 mM) were added to the postmitochondrial supernatant, collected from intact mitochondria or from mitochondria disrupted by either osmotic shock or digitonin treatment (Fig. 4). In intact mitochondria, there was no FAD appearance, but conversion of FMN to Rf was observed (4.2 pmol in 15 min of incubation; Fig. 4A). This was presumably due to FMN hydrolase activity (EC 3.1.3.2) [42,45]. After disruption of the mitochondrial membranes, FAD synthesis, as well as FMN hydrolysis, was seen in the mitochondria disrupted by digitonin treatment (8.7 pmol FAD; Fig. 4A), thus proving the existence of FADS activity in the mitochondrial inner compartment. As a control (Fig. 4B), disruption of the mitochondrial inner membrane integrity was evaluated by measuring both the latency of the matrix marker enzyme FUM and the release of a 58 kDa protein [mitochondrial chaperone 60 (Cnp60p)], revealed by western blotting.

Taken together, these results strongly favour the existence of (at least) two transport systems involved

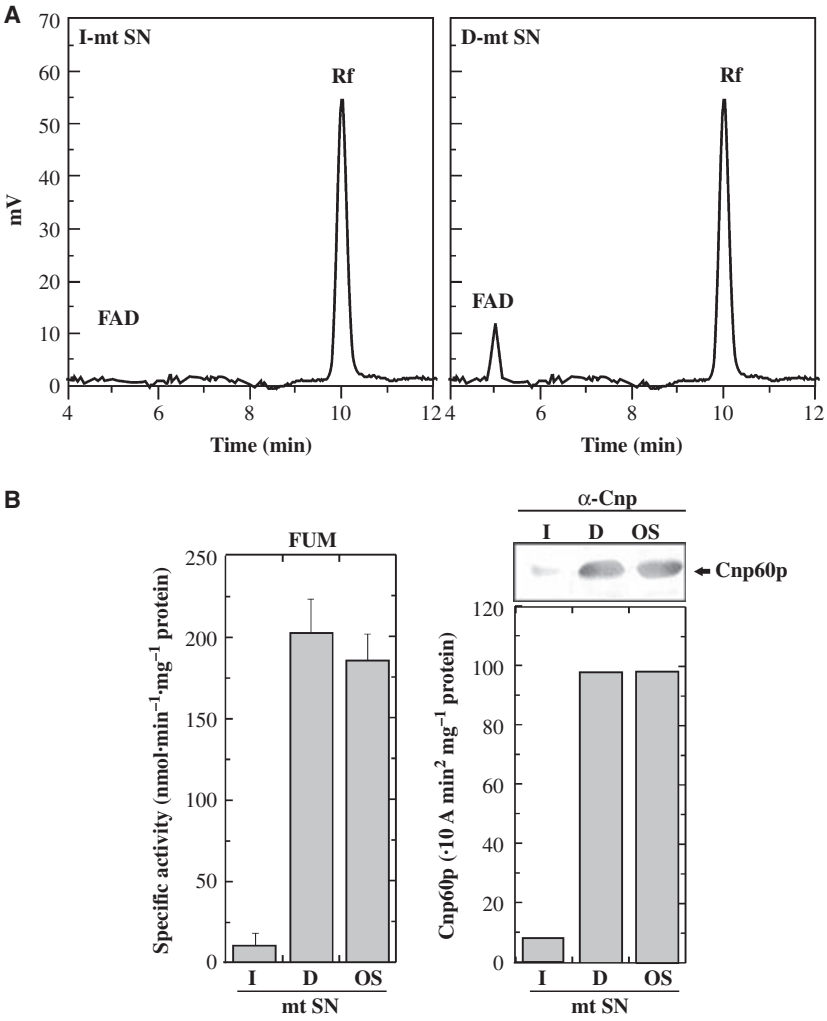

Fig. 4. FMN metabolism in the postmitochondrial supernatant. Postmitochondrial supernatants (0.1–0.2 mg) were collected from either intact (I-mt SN), digitonin-solubilized (D-mt SN) or osmotically shocked (OS-mt SN) TBY-2 mitochondria, as described in Experimental procedures. (A) I-mt SN and D-mt SN were incubated at 37 °C for up to 15 min with FMN (1 μM) and ATP (1 mM) in 500 μL of 50 mM Tris/HCl (pH 7.5), and the flavin amount was determined in neutralized perchloric acid extracts by HPLC. (B) FUM activity and the amount of Cnp60p, detected with α-Cnp, were measured in the I-mt SN, D-mt SN and OS-mt SN. Values, reported in the histogram, are the mean (± SD) of three replicates performed using the same mitochondrial preparations.

in Rf uptake into/FAD export out of mitochondria, as already observed in mitochondria from mammals and yeasts [29–32]. Moreover, the data here reported imply the existence of intramitochondrial enzymes that allow for FMN and FAD synthesis starting from exogenous Rf and endogenous ATP.

RK and FADS – Rf-metabolizing activities in TBY-2 mitochondria

In a further set of experiments, TBY-2 mitochondria were ruptured by osmotic shock or solubilized by detergent treatment (i.e. digitonin or Lubrol PX). Ruptured TBY-2 mitochondria were incubated for different incubation times (ranging from 1 to 60 min) at 37 °C with ATP (1 mM) and either Rf or FMN in the presence of $MgCl_2$ (5 mM) (Fig. 5). The amounts of FAD, FMN and Rf in the neutralized perchloric acid extracts of the suspension were measured by HPLC. Data were subtracted for endogenous FAD and FMN contents, which were equal to 243 ± 55 and 172 ± 16 pmol·mg^{-1} protein, respectively, in the experiment reported in Fig. 5. A control was also set up so that the endogenous flavin cofactor content remained constant during the incubation period (data not shown).

With Rf (0.5 μM) as a substrate, FMN rapidly appeared in the mitochondrial suspension according to the existence of RK activity (Fig. 5A). The time course of FMN synthesis was described by a pseudo-first-order rate equation in which the amount of FMN increased linearly with time up to 773 pmol·mg^{-1} protein, at a rate equal to 1.1 nmol·min^{-1}·mg^{-1} protein. FMN synthesis was accompanied by the appearance of a small amount of FAD, at a rate of 4.5 pmol·min^{-1}·mg^{-1} protein. The dependence of FMN synthesis rate on the substrate concentration showed saturation characteristics with a sigmoidal shape. Data

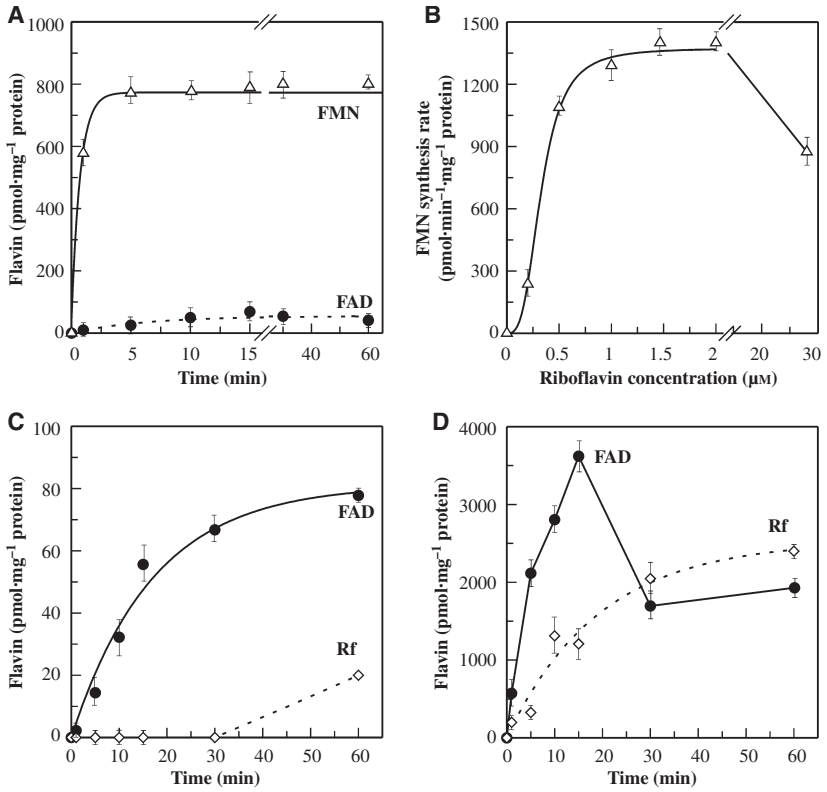

Fig. 5. Rf and FMN metabolism in osmotically shocked TBY-2 mitochondria. Osmotically shocked TBY-2 mitochondria (0.1–0.2 mg) were incubated at 37 °C in 500 μL of 50 mM Tris/HCl (pH 7.5) supplemented with ATP (1 mM) and $MgCl_2$ (5 mM), in the absence or presence of either Rf or FMN. At the appropriate times, the reaction was stopped, and Rf (◇), FMN (△) and FAD (●) contents were determined in neutralized perchloric acid extracts by HPLC, corrected for endogenous flavin content. (A) Time course of FMN and FAD synthesis after addition of 0.5 μM Rf. (B) Dependence of the rate of FMN synthesis on Rf concentrations. (C,D) Time courses of FAD synthesis and Rf appearance after addition of either 1 μM or 50 μM FMN. Values are the mean of three replicates (± SD) performed using the same mitochondrial preparations.

fitting according to allosteric kinetics (Eqn 2) gave a Hill coefficient equal to 3.1, and $S_{0.5}$ and V_{max} values equal to 0.32 ± 0.12 μM and 1375 ± 45 pmol·min^{-1}·mg^{-1} protein, respectively (Fig. 5B). The FMN synthesis rate was inhibited when the Rf concentration was raised to 30 μM (Fig. 5B), and totally inhibited when either Mg^{2+} was omitted or EDTA (1 mM) was added to the incubation mixture (data not shown).

With FMN (1 μM) as a substrate, mitochondrial FAD synthesis was observed (Fig. 5C). The time course of conversion of FMN to FAD was described by a pseudo-first-order rate equation in which the amount of FAD increased linearly with time up to 81 pmol·mg^{-1} protein at a rate equal to 5 pmol·min^{-1}·mg^{-1} protein. Following 1 h of incubation, FMN hydrolysis was detected, with 20 pmol·mg^{-1} protein of Rf being present in the mitochondrial suspension. When the FMN concentration was increased to 50 μM (Fig. 5D), the amount of FAD increased almost linearly in the first 10 min of the reaction, at a rate of 413 pmol·min^{-1}·mg^{-1} protein. The amount of FAD reached a maximum value of 3600 pmol·mg^{-1} protein within 15 min of incubation. Prolonging the incubation time resulted in a significant decrease in the amount of FAD. With prolonged incubation, the hydrolytic process became relevant, causing a progressive increase in Rf at a rate equal to 131 pmol·min^{-1}·mg^{-1} protein. Because of FMN hydrolysis, a correct estimation of the kinetic parameters of FADS in such a 'crude' mitochondrial extract was not possible. Both FAD formation and FMN hydrolysis were prevented by omitting Mg^{2+} (data not shown).

The amount of endogenous FAD and the rate of FAD formation in solubilized mitochondria were also measured in a continuous spectrophotometric assay by using the apoenzyme of D-amino acid oxidase (EC 1.4.3.3) in a coupled enzymatic assay, described in Fig. 6 and in more detail in [30,32]. A typical experiment is reported in Fig. 6B. Solubilized mitochondria (Fig. 6B, dotted line) were incubated first in the absence of the FADS substrate pair (−FMN, −ATP). A decrease in NADH absorbance was observed, corresponding to 246 pmol·mg^{-1} protein of mitochondrial endogenous FAD, which is expected to be loosely bound and/or not bound to protein. The value here tallies pretty well with the value obtained from HPLC measurements (Table 1). Solubilized mitochondria were then ultrafiltered prior to the assay (Fig. 6B, dashed and continuous lines), with the aim of removing endogenous intramitochondrial flavins that could inhibit FAD synthesis. Consistently, no FAD could be detected in the absence of the FADS substrate pair

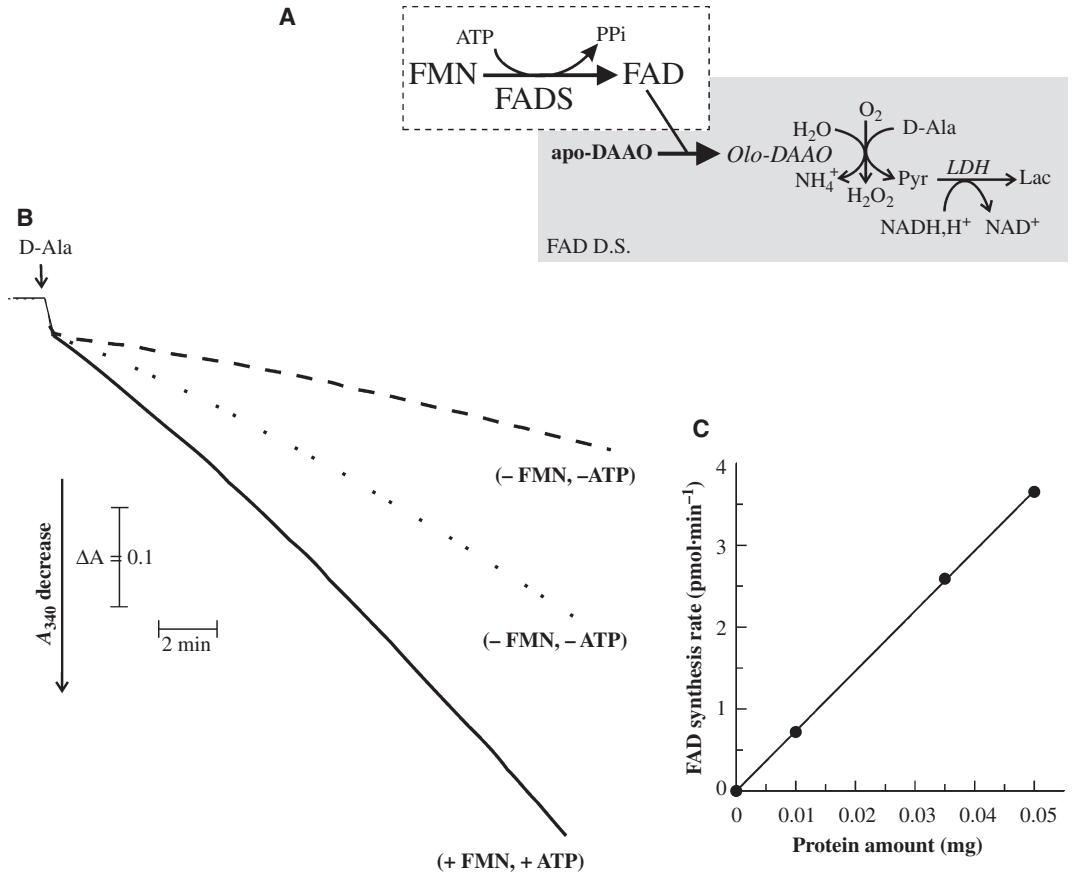

Fig. 6. Enzymatic evidence of FAD synthesis in solubilized TBY-2 mitochondria. The amount of FAD was enzymatically assayed in Lubrol PX-solubilized TBY-2 mitochondria, as shown in (A) and described in Experimental procedures. An aliquot of solubilized TBY-2 mitochondria was depleted of free flavins and other low molecular mass molecules by ultrafiltration procedures. (B) Solubilized (dotted line) or ultrafiltered solubilized (dashed and continuous lines) TBY-2 mitochondria were incubated with or without FAD substrate pairs (FMN 1 μM and ATP 1 mM) for 15 min at 37 °C in 100 μL of 50 mM Tris/HCl (pH 7.5) supplemented with $MgCl_2$ (5 mM). (C) The dependence on protein amount of the rate of FAD synthesis in ultrafiltered solubilized TBY-2 mitochondria is reported.

(Fig. 6B, dashed line, −FMN, −ATP) or in the absence of either FMN or ATP alone. Upon incubation of ultrafiltered solubilized mitochondria with both FMN and ATP (Fig. 6B, continuous line, +FMN, +ATP), FAD synthesis was observed with a rate equal to 2.5 pmol·min^{-1}. This rate was linearly related to the amount of the mitochondrial protein used (74 pmol·min^{-1}·mg^{-1} protein; Fig. 6C), corresponding to a total mitochondrial activity of 488 pmol·min^{-1} at 1 μM FMN. When the ultrafiltration procedure was omitted, the rate of formation of FAD by solubilized mitochondria (+FMN, +ATP, data not shown) was about 10-fold lower, and therefore in broad agreement with the rate calculated via HPLC (Fig. 5C).

From the results obtained using TBY-2 mitochondria, we could not establish whether the mitochondrial RK and FADS activities reside in a single bifunctional enzyme, such as RibC in *Bacillus subtilis* [33], or whether they are two distinct enzymes as in other eukaryotes. To overcome this problem, we searched for conditions in which the two activities might be physically separated. Therefore, RK and FADS were checked in a mitochondrial-soluble fraction (S_{fr}) and in a mitochondrial membrane-enriched fraction (M_{fr}), obtained as described under Experimental procedures, and compared with those of FUM and SDH, used as matrix and inner mitochondrial membrane marker enzymes, respectively (Fig. 7). When RK substrate pairs were added to S_{fr} (Fig. 7A) or M_{fr} (Fig. 7A′), 3.5 and 0.6 pmol of newly synthesized FMN were determined respectively in the two fractions. About 85.5% of total RK activity was recovered in the S_{fr}, in fairly good accordance with the matrix marker enzyme FUM activity (the total activity recovered in the S_{fr} being equal to 82.5% in Fig. 7C). When the FADS substrate pair was used, 1.5 and 2.1 pmol of newly

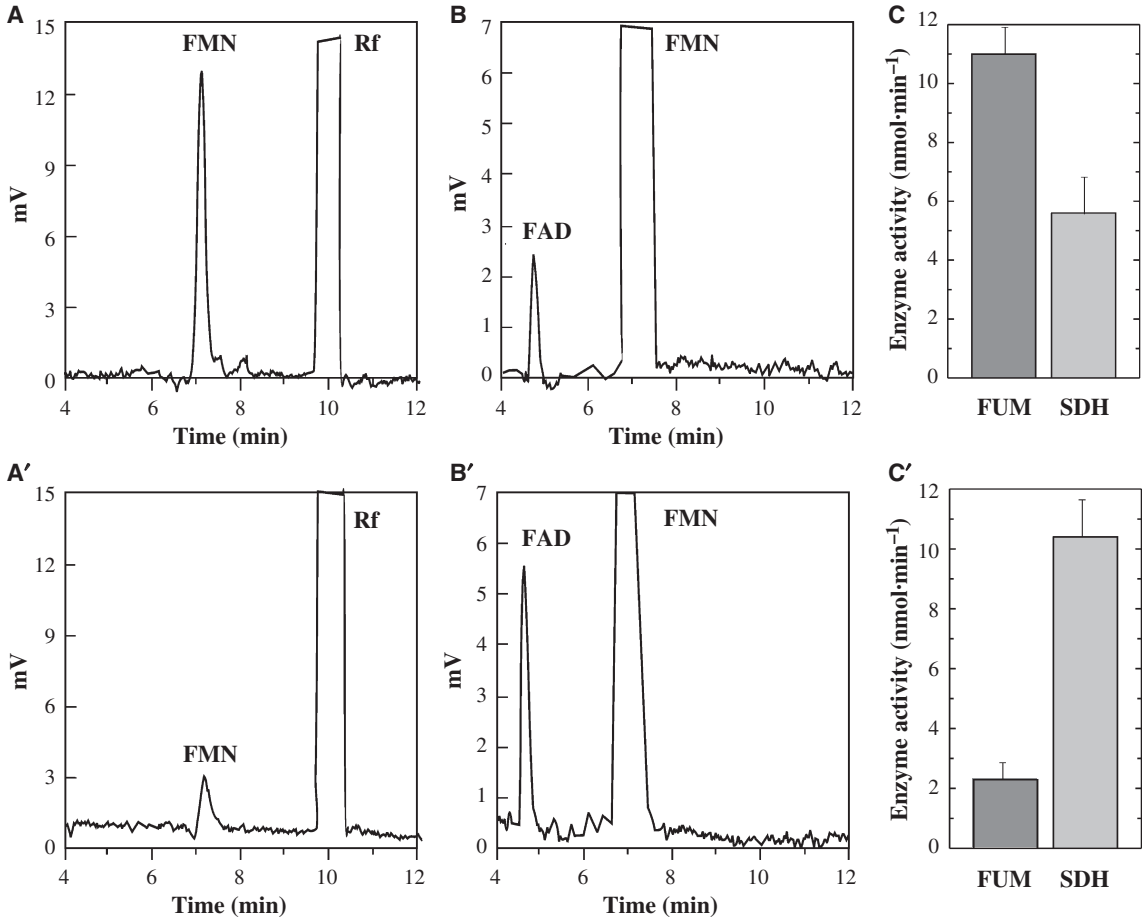

Fig. 7. Distribution of the RK and FADS activities in the mitochondrial subfractions. Soluble (S_{fr}) (A–C) and membrane-enriched (M_{fr}) (A′–C′) fractions, obtained from TBY-2 mitochondria as described in Experimental procedures, were assayed for mitochondrial RK (A,A′) and FADS (B,B′), as in Fig. 5. As a control in the same fractions, the total activities of FUM and SDH (C,C′), used as mitochondrial matrix and inner membrane marker enzymes, were determined.

synthesized FAD were determined, respectively, in the S_{fr} (Fig. 7B) and in the M_{fr} (Fig. 7B′). With regard to total FADS activity, about 60% was recovered in the M_{fr}, in fairly good agreement with the 65% recovery of the SDH enzymatic activity (Fig. 7C′). Taken together, these findings show that mitochondrial RK and FADS activities reside in distinct enzymes that are physically separated in the S_{fr} and M_{fr}, respectively.

Discussion

Because of its importance in energetic metabolism, as well as in human and animal nutrition [1,17,46,47], the biosynthetic pathway of several vitamins and coenzymes is one of the more interesting topics for biochemical analysis in plants.

The experiments described here deal with the mechanism by which plant mitochondria obtain their own flavin cofactors, starting from Rf synthesized *de novo* in the plastids [17]. To this end, use was made of bioenergetically active and highly purified mitochondria prepared starting from protoplasts of TBY-2 cells, which can take up externally added Rf via saturable mechanisms that operate at different concentration ranges and have different uptake efficiencies.

At the lower concentration of Rf used (0–3.0 μM), which roughly corresponds to the physiological concentration of the vitamin measured in protoplasts, no flavins accumulate in the organelle. Conversely, FAD is the only flavin cofactor detected in the postmitochondrial supernatant. These results are in line with the existence of both a mitochondrial FAD synthesis pathway and a mitochondrial FAD exporter, as in rat liver and *S. cerevisiae* mitochondria [30–32]. Indeed, the rate of FAD appearance depends on up to five events: Rf uptake, conversion into FMN, conversion of FMN

into FAD, FAD export, and inhibition of FMN synthesis. At least under our *in vitro* conditions, at low vitamin concentrations, Rf uptake is expected to be the rate-limiting step of the overall process, as no intermediates accumulate. Thus, the kinetic parameters of the Rf transporter are calculated from those describing FAD appearance in the postmitochondrial supernatant.

When higher Rf concentration are used (10–30 μM), Rf transport rate increases, causing high Rf concentrations inside the limited space of the mitochondrial matrix. Under this condition, mitochondrial RK is completely inhibited (see below and [30–32]). This results in Rf accumulation in the organelle and no FAD export in the postmitochondrial supernatant. In this concentration range, the sigmoidal shape might be characteristic of the Rf transporter itself. Whether or not, *in vivo*, such high concentrations of Rf could physiologically be realized, it might still be a possibility in microcompartments of the intramembrane space during the recycling hydrolytic pathway of mitochondrial FAD [45].

Further experiments are in progress to identify suitable inhibitors of flavin transport across mitochondrial membrane and to further characterize and to identify the mitochondrial Rf uptake and FAD export transporter(s).

At present, we have no putative candidate gene encoding any mitochondrial Rf transporter. In fact, FASTA searches (http://www.ebi.ac.uk/Tools/fasta), using as query sequences the first identified prokaryotic Rf transporter, *YpaA* from *B. subtilis* [48], the first identified eukaryotic plasma membrane Mch5p from *S. cerevisiae* [49], and the novel identified human and rat riboflavin plasma membrane transporters (hRFT1 and rRFT1) [50], revealed no sequence homologs in either *A. thaliana* or *Oryza sativa*. In contrast, FASTA searches revealed more than 30 sequence homologs of the mitochondrial FAD exporter (Flx1p) from *S. cerevisiae* [32,51]. Among these, a mitochondrial localization is predicted for two uncharacterized proteins encoded by *At1g25380* and *At2g47490* in *A. thaliana* (see The Arabidopsis Information Resource database, TAIR, http://www.arabidopsis.org), and for the uncharacterized protein encoded by *Os03g0734700* in *O. sativa* (see UniProt/TrEMBL database, http://www.ebi.ac.uk/trembl). The hypothesis that these proteins are orthologs of Flx1p is at the moment under investigation.

In this article, we also give the first experimental evidence for the existence of a FADS in plant mitochondria, which catalyses FAD synthesis from FMN and ATP, and we confirm the existence of a mitochondrial RK [26,30–32,41,43]. Using ruptured mitochondria, functional characterization of the mitochondrial RK and FADS was performed (Figs 5–7). Both of the TBY-2 mitochondrial FAD-forming enzymes are activated by $MgCl_2$, a feature common to other RK(s) and FADS(s) previously characterized from prokaryotic and eukaryotic sources [20–22,27,28,33–42].

The dependence of the rate of FMN synthesis on Rf concentration shows saturation characteristics with a sigmoidal shape. The $S_{0.5}$ value of RK is in the same order of magnitude as the K_m measured for the RK partially purified from the plant *Solanum nigrum* [39], and one order of magnitude higher than the K_m value of the bifunctional AtFMN/FHy enzyme from *A. thaliana* [42]. Earlier enzymological studies [52] and latest structural data [24] suggest that the activity of RK(s) is largely regulated by the relative concentrations of substrates/products, as well as by specific interactions with other regulators (i.e. bivalent cations).

A detailed kinetic study of FADS is prevented by the rapid conversion of FMN to Rf, stimulated by $MgCl_2$. This is expected to be due to an FMN hydrolase activity, present in the ruptured TBY-2 mitochondria. Plant FMN hydrolases have been recently assayed in both chloroplast and mitochondrial extracts from pea. Owing to this high FMN-hydrolysing activity, no natural FADS activity has been detected before in plants [43]. We succeeded in detecting FADS activity in ruptured TBY-2 mitochondria by HPLC and then enzymatically. The approximately 100-fold increase in the initial rate of FADS production, which we have measured with increasing FMN concentrations from 1 to 50 μM (Fig. 5C,D), is consistent with the K_m values (18–20 μM) determined for the monofunctional recombinant FADS(s) [43]. It can be argued that in ruptured mitochondria, unlike in intact organelles, FMN appears and its concentration exceeds that of FAD (compare Figs 5 and 3). The simplest explanation for this is based on the existence of 'channelling' between RK and FADS in intact mitochondria, which is lost in ruptured mitochondria.

Indeed, our studies revealed that RK and FADS are two physically separated enzymes, one being found in the mitochondrial matrix and the other being membrane associated.

The genes encoding organellar RK(s) remains unidentified. The products of *AtRibF1* and *AtRibF2*, homologs of the bifunctional bacterial RibC and recently characterized in *A. thaliana*, perform only FADS activity. Conversely, AtFMN/FHy is the cytosolic RK [42].

Our fractionation studies reveal that mitochondrial FADS activity in TBY-2 mitochondria represents

about 3% of the total activity determined in the protoplasts, as estimated by comparison with the distribution of the marker enzyme FUM, and assuming that the highest amount of FUM activity is present in the mitochondrial fraction. Conversely, FADS activity is maximally present in plastids (its specific activity at 1 μM FMN is equal to 466 pmol·min^{-1}·mg^{-1} protein, i.e. 23% of the total activity determined in the protoplasts); the same distribution is obtained for the plastid marker enzyme PGI. These results tally well with confocal microscopy studies carried out on *A. thaliana* protoplasts transformed with enhanced green fluorescent protein (EGFP)–AtRibF1 or EGFP–AtRibF2 [43]. The hypothesis for the localization of FADS (AtRibF1 and AtRibF2) isoforms in mitochondria cannot, moreover, be ruled out on the basis of bioinformatics (see TAIR). Whether and how it can be achieved remains to be established.

The final picture emerging is that of cross-talk between plastids, cytosol and mitochondria during flavin cofactor biosynthesis, which completes the scheme reported in [43]. Rf is synthesized *de novo* in plastids [17] and converted therein into FMN and FAD [41,43]. Alternatively, Rf can be exported into the cytosol and taken up by mitochondria, where an autonomous FAD-forming pathway is expected to respond to the demand for nascent apoflavoprotein deriving from outside [53–55]. Mitochondrial FAD in plants, as well as in yeasts [18,31] and mammals [30], can also be exported to the cytosol. Whether or not the exported FAD participates in regulating the expression of nascent mitochondrial flavoproteins, as in yeast [18], remains an intriguing question for future analysis.

Experimental procedures

Materials

All reagents and enzymes were from Sigma-Aldrich (St Louis, MO, USA). Mitochondrial substrates were used as Tris salts at pH 7.0. Solvents and salts used for HPLC were from J. T. Baker (Deventer, The Netherlands).

Cell culture

TBY-2 cells were routinely propagated and cultured at 27 °C, essentially as described in [13].

Protoplast, mitochondria and plastid preparation

Protoplasts were obtained from TBY-2 cells (50 g) washed with a preplasmolysis buffer (0.65 M mannitol and 25 mM Tris/Mes, pH 5.5) and treated with Caylase (Cayla, Toulose, France) and pectinase (Sigma-Aldrich), as described in [13]. Intact purified mitochondria and plastids were obtained by protoplast fractionation and lysis, followed by differential centrifugation and by a self-generated Percoll density gradient (0–40%), as described in [13]. Protoplasts, mitochondria and plastids were ruptured by osmotic shock by resuspending them in a washing medium without mannitol (hypotonic medium) or by treatment with the detergent Lubrol PX (0.3 mg·mg^{-1} protein) or digitonin (0.4 mg·mg^{-1} protein). Postmitochondrial supernatant was collected from either intact, osmotically shocked or digitonin-treated mitochondria after centrifugation at 15 000 ***g*** for 5 min. Mitochondria ruptured by osmotic shock were centrifuged at 20 000 ***g*** for 30 min to separate S_{fr} and M_{fr}, as in [13]. The protein concentration was assayed according to Bradford [56].

Mitochondrial integrity and oxygen uptake measurements

The intactness of mitochondrial inner membranes was checked by measuring the release of the matrix FUM, as in [57]. Oxygen uptake measurements were carried out at 25 °C using a Gilson 5/6 oxygraph with a Clark electrode. Mitochondria (0.1 mg) were added to 1.5 mL of respiration medium containing 0.3 M mannitol, 10 mM Hepes, 5 mM $MgCl_2$, 10 mM KCl and 0.1% BSA (the pH of the medium was adjusted to 7.2 with NaOH). NADH (1 mM) or succinate (5 mM) was used as a respiratory substrate. The rate of oxygen uptake, measured as the tangent to the initial part of the progress curve, was expressed as nmol O_2·min^{-1}·mg^{-1} protein.

Rf uptake and metabolism

Freshly isolated mitochondria (0.1–0.2 mg of protein) were incubated at 2 °C in 500 μL of transport medium consisting of 0.3 M mannitol, 10 mM Hepes and 5 mM $MgCl_2$ (the pH was adjusted to 7.5 with NaOH). One minute later, Rf was added. At the appropriate time, the uptake reaction was stopped by rapid centrifugation. Rf, FMN and FAD contents of supernatants and pellets were measured in aliquots (5–80 μL) of neutralized perchloric acid 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 [32]. The amount of flavin actually taken up into the organelle was calculated after correction was made for molecules present in the adherent space and/or nonspecifically bound to the membranes, as described elsewhere [32].

RK and FADS activity assay

Detergent-solubilized or osmotically shocked mitochondria or postmitochondrial supernatants (0.1–0.2 mg) were preincubated at 37 °C for 1 min in 500 μL of a medium consisting of 50 mM Tris/HCl (pH 7.5); where indicated, 5 mM $MgCl_2$ or 1 mM EDTA was added. Either Rf or FMN (at the indicated concentrations) and ATP (1 mM) were added in order to assay for RK or FADS activity, respectively. At the appropriate time, 50 μL aliquots were taken, extracted with perchloric acid, and neutralized. Rf, FMN and FAD were analysed using HPLC (see above). The amount of FAD was also measured enzymatically by using the FAD-detecting system, as described in [30,32]. Briefly, the amount of FAD was determined by revealing the reconstituted holo-D-amino acid oxidase (D-AAO) activity derived from FAD binding to the apo-D-AAO, using D-alanine (25 mM) as substrate. The rate of NADH oxidation in the L-lactate dehydrogenase-coupled reaction was followed spectrophotometrically at 340 nm by means of a Perkin Elmer λ19 spectrophotometer, and calculated as a tangent to the linear part of the progress curve. This rate was proven to be proportional to FAD concentration. Calibration curves were obtained by using standard FAD solutions, and corrections were also made to account for the inhibition due to FMN and ATP added to the reconstitution assay.

Western blotting

Proteins from protoplasts, mitochondria and plastids were separated by SDS/PAGE [58] and transferred as in [32]. The immobilized proteins were incubated with a 2000-fold dilution of either a polyclonal antibody against FAD covalently bound to proteins (i.e. α-FAD, a kind gift from R. Brandsch, Freiburg, Germany; for details see [32]) or an antiserum against the chaperonin (i.e. α-Cnp, a kind gift from C. Indiveri, Università della Calabria, Calabria, Italy). α-FAD- and α-Cnp-immunoreactive materials were visualized with the aid of a secondary alkaline phosphatase-conjugated anti-rabbit IgG. Quantitative evaluations were carried by densitometric analysis using IMAGEQUANT 5.2 Software (Molecular Dynamics, Sunnyvale, CA, USA).

Other enzymatic assays

SDH and PGI activities were measured as in [18]. Glutamate synthase activity was determined by measuring the decrease of absorbance at 340 nm due to NADH oxidation in a reaction mixture containing 50 mM sodium phosphate buffer (pH 7.5), 10 mM 2-oxoglutarate, 10 mM glutamine and the biological sample, essentially as described in [59]. ADH activity was tested by measuring the increase in absorbance at 340 nm due to NAD^+ reduction after addition of 20% ethanol in a reaction mixture containing 50 mM Tris/HCl (pH 9) and 0.867 mM NAD^+ [44].

Kinetic data analysis

Data fitting was performed according to either the Michaelis–Menten equation:

$$v = V_{max}S/(K_m + S) \quad (1)$$

or the allosteric kinetics equation

$$v = V_{max}S^n/(K_m + S^n) \quad (2)$$

where $S_{0.5} = \sqrt[n]{K_m}$.

To fit the experimental data and to obtain estimates of the kinetic parameters, use was made of the GRAFIT software (Version 3.00, 1992, by R. J. Leatherbarrow, Erithacus Software, Horley, UK).

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