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Reduction of Exogenous Flavins and Mobilization of Iron from Ferritin by Isolated Mitochondria

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Abstract

The release mechanism for ferritin iron and the nature of the compound(s) which donate iron to the mitochondria are two important problems of intracellular iron metabolism which still await their solution. We have previously shown that isolated mitochondria reduce exogenously added flavins in a ubiquinol-flavin oxidoreductase reaction at the C-side of the inner membrane and that the resulting dihydroflavins function as reductants in mitochondrial mobilization of iron from ferritin (Ulvik, R. J., and Romslo, I. (1981). *Biochim. Biophys. Acta* **635,** 457–469). In the present study it is shown that the rate at which iron is removed from ferritin depends on the capability of the flavins to penetrate (1) the mitochondrial outer membrane and (2) the intersubunit channels of the ferritin protein shell. Intact mitochondria reduce flavins at rates which decrease in the following order: riboflavin $\gg$ FAD $>$ FMN. The ferritin iron mobilization rates decrease in the order of riboflavin $>$ FMN $>$ FAD. The results are further support for the operation of a flavin-dependent mitochondrial ferrireductase, and strengthen the suggested role for ferritin as a donor of iron to the mitochondria.

Key Words: Ferritin; mitochondria; flavin; iron mobilization.

Introduction

Deposition of iron in ferritin is a reversible process which means that at increased metabolic needs, iron can be removed from ferritin to the cytoplasma (Munro and Linder, 1978). Whether ferritin plays a role in transport and distribution of iron within the cell is a question subject to current discussion and investigation (Mazur and Carleton, 1963; Speyer and Fielding, 1979; Nunez *et al.*, 1980; Romslo, 1980). Of particular interest is a possible function for ferritin as an intermediate in the transport of iron from the plasma membrane across the cytosol to the mitochondria which, owing to

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their central position in the biosynthesis of heme, receive a greater portion of the iron accumulated by the cell (Jones and Jones, 1969; Nunez *et al.*, 1978; Ulvik and Romslo, 1978, 1979; LaCross and Linder, 1980; Ofer *et al.*, 1981).

Ferritin loses its iron either to chelators which have a high affinity for iron, or by the action of reducing agents (Harrison *et al.*, 1977). Reductive release of iron most likely depends on a small-molecular-weight reductant capable of penetrating through or within the intersubunit channels of the ferritin protein shell (Dognin and Crichton, 1975; Harrison *et al.*, 1980). The highest iron mobilization rates have been found with free dihydroflavins (Sirivech *et al.*, 1974; Jones *et al.*, 1978), and therefore release of iron from ferritin is believed to involve the operation of one or more flavin-reductase reactions in the cell. Of physiological interest is the suggested function of xanthine oxidase (Green and Mazur, 1957; Mazur *et al.*, 1958) and of NAD(P)H-FMN oxidoreductases which may be located in cytosol (Osaki and Sirivech, 1971; Sirivech *et al.*, 1977) or in the microsomes (Zaman and Verwilghen, 1979). Because of conflicting and insufficient evidence, however, the importance of the interaction of these enzymes with cellular iron metabolism remains to be confidently established (Grace *et al.*, 1970; Sirivech *et al.*, 1977; Romslo, 1980).

Recently, we have characterized a ubiquinol-FMN oxidoreductase reaction at the C-side of the inner membrane of isolated mitochondria (Ulvik and Romslo, 1981). $FMNH_2$ generated in this reaction removes iron from ferritin molecules adherent to the surface of the mitochondria, and the released iron is readily incorporated into heme (Ulvik *et al.*, 1981; Ulvik, 1981, 1982). These studies suggest a role for ferritin as donor of iron to the mitochondria. In the cell, flavin is accumulated as riboflavin which is rapidly converted to FMN and further to FAD by the cytosolic enzymes flavokinase and FAD-pyrrophosphorylase, respectively (McCormick, 1975). Accordingly, FAD is the dominating form mounting to approximately 80–90% of the total flavin content with 10–18% as FMN and 1–2% as riboflavin (McCormick, 1975). From a physiological point of view, it is therefore of interest to clarify whether the mitochondrial FMN-dependent ferrireductase also operates with the alternative flavin forms.

In the present work the effect of riboflavin, FMN, and FAD as reductants in the mobilization of iron from ferritin by isolated mitochondria has been compared. In particular, the significance of the mitochondrial membranes to the generation of dihydroflavins by the mitochondria has been studied.

Materials and Methods

Rat liver mitochondria were prepared as described previously (Romslo and Flatmark, 1973). Mitoplasts were prepared by the digitonin method

(Chan *et al.*, 1970) with adenylate kinase activity less than 5% compared to intact mitochondria (Ulvik and Romslo, 1981). The functional integrity of the mitochondria was tested by measuring the respiratory control ratio with ADP using succinate as substrate. Only mitochondria with respiratory control ratios greater than 3.0 were used for experiments.

Mobilization of Iron from Ferritin

Mitochondria, approximately 2 mg protein/ml, were incubated in a medium containing 0.25 M sucrose, 10 mM Hepes[2] buffer (pH 7.40), 1 mM succinate, 40 μM bathophenanthroline, 0.30 μM ferritin (0.6 mM iron), and 32 μM riboflavin, FMN, or FAD as indicated in the legends to the figures. The temperature of incubation was 30°C. Autoxidation of dihydroflavin was omitted by keeping the oxygen concentration of the medium at near anaerobiosis (Ulvik and Romslo, 1981). It has been shown previously that the structure, oxidative phosphorylation, and energy-conserving properties of the mitochondria are maintained during near-anaerobic incubation (Ulvik, 1981).

Release of iron from ferritin was determined by measuring the change in absorbance Δ ($A_{530nm} - A_{560nm}$) owing to the production of the iron-bathophenanthroline complex (Ulvik and Romslo, 1979). Incubation was carried out in an Aminco DW 2 UV/VIS spectrophotometer equipped with a vibrating platinum electrode to control the oxygen concentration of the medium.

Reduction of Exogenous Flavins

Reduction of exogenous flavins by the mitochondria was determined from the decrease in absorbance Δ ($A_{450nm} - A_{530nm}$) = 10.3 $mM^{-1} \times cm^{-1}$ (Green and Wharton, 1963; Massey and Hemmerich, 1980; Ulvik and Romslo, 1981). The experimental conditions were as described for the mobilization of iron from ferritin except that ferritin and bathophenanthroline were omitted.

Chemicals

Bathophenanthroline, bathophenanthroline sulfonate, riboflavin, FMN, FAD, Triton X-100, and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) were obtained from the Sigma Chemical Co. (St. Louis, Missouri, USA). Ferritin (equine spleen, A grade, twice crystallized, cad-

[2]*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

mium free) was from Calbiochem (Luzern, Switzerland). Other chemicals were of the highest purity commercially available. Double quartz-distilled water was used throughout.

Results

The capacity of the mitochondria to remove iron from ferritin in the presence of exogenous flavin was decreased in the order riboflavin > FMN > FAD (Figs. 1 and 3). Without flavin no iron was released from ferritin (Fig. 1). Steady-state iron mobilization rates were invariably preceded by a lag phase of a duration which depended on the size of the flavin molecules, i.e., the lag phase with FAD > FMN > riboflavin (Fig. 1). If the flavins were reduced by dithionite instead of mitochondria, the lag phase was shortened and the rate of iron mobilization from ferritin was significantly increased for all three flavins (Figs. 2 and 3). While the difference between riboflavin and FMN was essentially abolished with dithionite as reductant, FAD behaved principally as in the mitochondrial system with a longer delay to reach steady state and a slower iron mobilization rate than riboflavin and FMN (Figs. 2 and 3).

In intact mitochondria the rate of reduction of exogenous flavins decreased in the order riboflavin ≫ FAD > FMN, with 0.54, 0.30, and 0.22

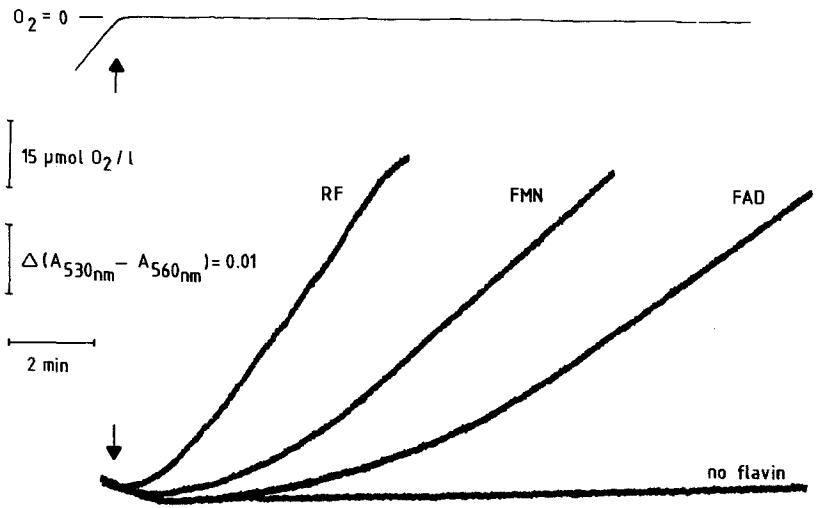

Fig. 1. Mobilization of iron from ferritin by mitochondria in the presence of exogenous riboflavin, FMN, or FAD. Experimental conditions as described in Materials and Methods. The oxygraphic trace at the top shows the oxygen concentration of the medium. Arrows indicate near anaerobiosis.

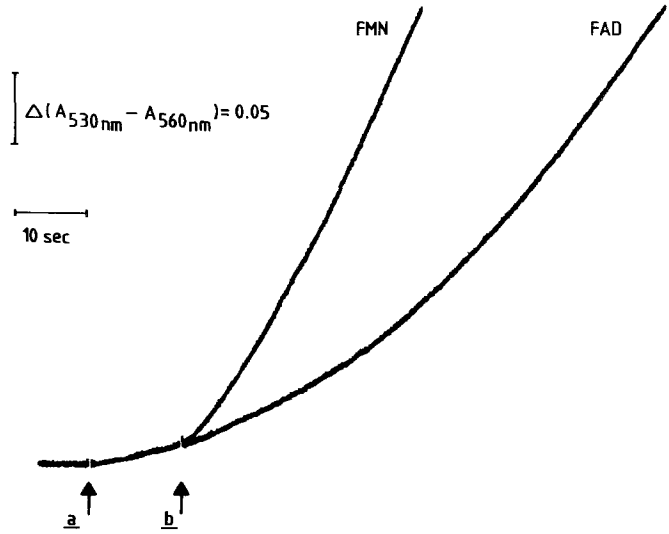

Fig. 2. Mobilization of iron from ferritin by $FMNH_2$ and $FADH_2$ reduced by dithionite. 0.30 μM ferritin was incubated in a sucrose/Hepes medium as in Fig. 1, containing 0.20 mM bathophenanthroline sulfonate. When indicated 1.4 mM dithionite (arrow a) and 40 μM FMN or FAD (arrow b) was added.

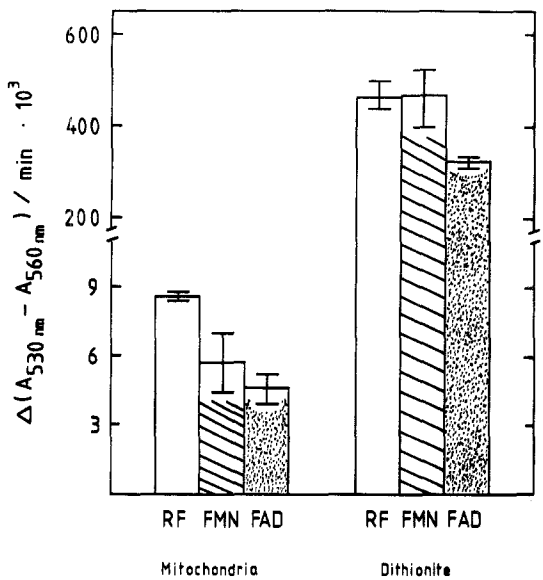

Fig. 3. Steady-state mobilization of iron from ferritin with riboflavin, FMN, or FAD reduced by mitochondria or dithionite. $n = 3$. Ranges are indicated.

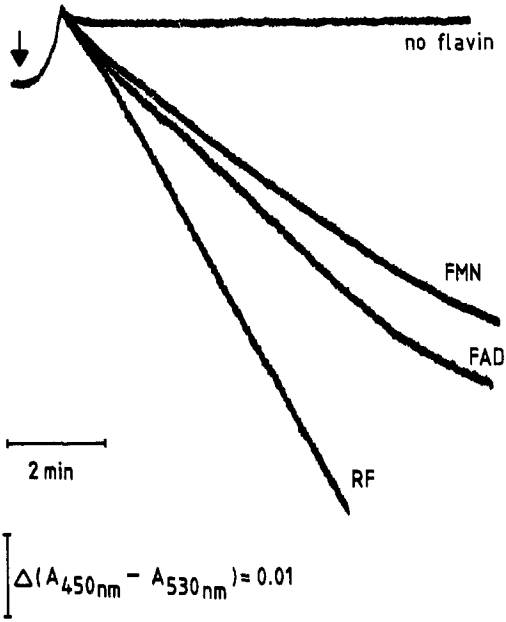

Fig. 4. Reduction of exogenous riboflavin, FMN, or FAD by intact mitochondria. Experimental conditions as in Fig. 1 except that ferritin and bathophenanthroline were omitted. Arrow indicates near anaerobiosis.

nmol dihydroflavin produced per minute per milligram mitochondrial protein, respectively (Figs. 4 and 5). In mitoplasts the steady-state reduction rate of FAD was markedly increased while no significant change occurred in the reaction rates of riboflavin and FMN when compared with intact mitochondria (Fig. 5). Mitochondria solubilized by Triton X-100 maintain their capability to reduce exogenous FMN unchanged within the first 4–5 min following solubilization (Ulvik and Romslo, 1981). As shown in Fig. 5 all three flavins were reduced at higher rates in solubilized mitochondria than in intact mitochondria and mitoplasts, with the reduction of riboflavin and FAD at similar rates and almost two times the rate of reduction of FMN. With KCl in the medium, intact mitochondria reduced riboflavin, FMN, and FAD at a much lower rate but in the same order as that found without KCl (Fig. 5).

Discussion

Reduction of exogenous flavin by isolated mitochondria is most likely carried out at the C-side of the inner membrane with ubiquinol as reductant.

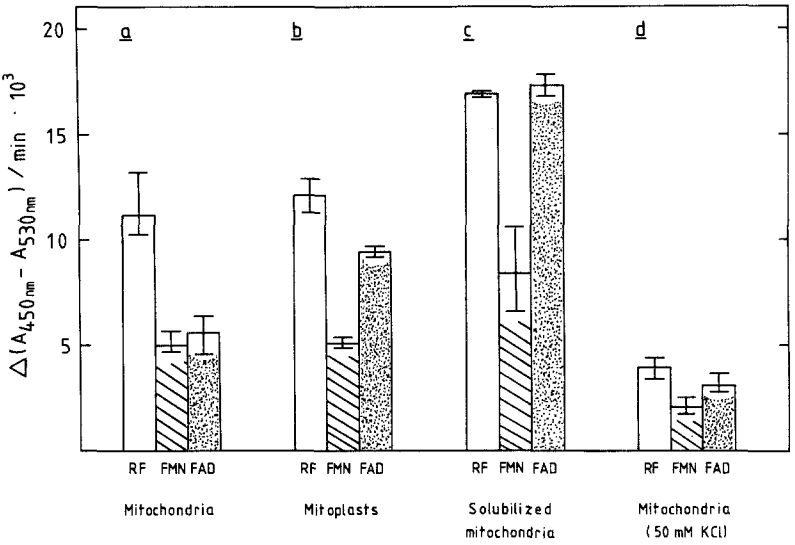

Fig. 5. Steady-state reduction of exogenous riboflavin, FMN, and FAD by intact mitochondria (a), mitoplasts (b), mitochondria solubilized by Triton X-100 (0.06%, v/v) (c), and intact mitochondria with 50 mM KCl present (d).

This process has been characterized in detail for FMN in previous studies (Ulvik and Romslo, 1979, 1981; Ulvik, 1982). To reach the catalytic oxidoreduction site, the flavin molecules must cross the outer membrane, probably through pores (Roos *et al.*, 1982). Due to its smaller molecular size, riboflavin would be expected to pass the outer membrane more easily than the larger molecules FMN and FAD. In accordance with this assumption is the finding that the rate of reduction of riboflavin by intact mitochondria is about two times higher than that of FMN and FAD, and the fact that while the rate of reduction of riboflavin in mitoplasts is unchanged, reduction of FAD is markedly increased (Figs. 4 and 5). The essentially unchanged rate of reduction of FMN in mitoplasts compared to intact mitochondria may be explained by its negatively charged phosphate group which by generating electrostatic forces interferes with the interaction between the FMN molecule and the catalytic site in the intact as well as the solubilized inner membrane. The permeability barrier limiting the admittance of flavin molecules to the catalytic site in intact mitochondria is removed when the membrane integrity is lost by solubilization. This is evident from the significant increase in the reduction rates for all three flavins and from the finding that riboflavin and FAD are reduced at the same rates (Fig. 5).

The lower reduction rates obtained in the presence of K^+ may be a result of the positive effect of K^+ on the integrity of the inner membrane which,

therefore, more efficiently limits the interaction between the flavin molecules and ubiquinol (Ulvik and Romslo, 1981; Ulvik, 1981). Another possibility is interference of K^+ with the association of the electrodeficient isoalloxazine ring of the flavin molecule to negatively charged groups on the surface of the inner membrane. This is a loose binding which is thought to precede the reaction between flavin and ubiquinol (Ulvik and Romslo, 1981).

The rate of mobilization of iron from ferritin by flavins depends on the concentration of dihydroflavins and their ability to penetrate through or within the intersubunit channels of the ferritin molecule (Jones *et al.*, 1978; Harrison *et al.*, 1980). Diffusion of dihydroriboflavin, $FMNH_2$, and $FADH_2$ through the channels of ferritin is limited according to their molecular structure and size. Thus, when the flavins were instantaneously and completely reduced by dithionite, the time to reach steady-state reaction rate and the rate of mobilization of iron from ferritin decreased in the order riboflavin = FMN > FAD (Figs. 2 and 3). Similar results were obtained by Sirivech *et al.* (1974). It can be concluded that the rate with which iron is removed from ferritin in the mitochondrial flavin-dependent ferrireductase reaction is the resultant of the capability of the flavin molecules to overcome the permeability barrier set up by (1) the mitochondrial membranes, in particular the outer membrane and (2) the channels which pierce the protein shell of the ferritin molecule. In agreement with this concept is the order of ferritin-iron mobilization rates found in the mitochondrial system with riboflavin, FMN, or FAD as reductants (Fig. 3).

Fazekas and Sandor (1973) found that 90 min after injection of radioactive riboflavin into rats, 90% of the radioactivity was incorporated into FAD, 6% was incorporated into FMN, and about 4% remained as riboflavin. Subcellular fractionation of the liver showed that about 35% of the injected flavin was present in the mitochondria. On this background it is of considerable interest to find that both riboflavin, FMN, and, in particular, FAD, can be reduced by the mitochondria and function as intermediate reductants in the mobilization of iron from ferritin. An unanswered question is whether flavins in the cell appear in a form which allows participation in the iron mobilization process. Green and Mazur (1957) argued that ferritin iron was released through a reaction with reduced FAD of the xanthine oxidase, and a major role for the ferritin–xanthine oxidase system in the mobilization of iron from the liver and intestinal mucosa was suggested (Mazur *et al.*, 1958; Mazur and Carleton, 1965; Krenitsky and Tuttle, 1978). Later studies, however, have shown that only a small amount of the total ferritin iron (less than 1%) is released in the xanthine oxidase reaction (Sirivech *et al.*, 1974; Duggan and Streeter, 1973), and neither the intestinal absorption of iron (Davis and Deller, 1966) nor the mobilization of iron from ferritin in different tissues was significantly decreased when the xanthine oxidase activity was inhibited by

allopurinol (4-hydroxypyrazolo[3,4-*d*]pyrimidine) (Kozma *et al.*, 1967; Grace *et al.*, 1970; Sirivech *et al.*, 1977).

Thus, although there is substantial evidence in the literature for the function of flavins in reductive release of iron from ferritin, we still do not know how this process is carried out *in situ*. Apparently, a better understanding of this important problem depends on increased knowledge of flavin metabolism in the cell (Jones *et al.*, 1978).

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