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BIOGENESIS OF FLAVOPROTEIN AND CYTOCHROME COMPONENTS IN HEPATIC MITOCHONDRIA FROM RIBOFLAVIN-DEFICIENT RATS

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SUMMARY: Using difference spectrophotometry, measurements of succinate dehydrogenase activity, and SDS-polyacrylamide gels, the biochemical properties of hepatic mitochondria from riboflavin-deficient rats were monitored during recovery on riboflavin. [^{14}C]Riboflavin was incorporated into four mitochondrial flavoproteins having covalently bound flavin coenzyme. Alterations in cytochromes, especially cytochrome oxidase, and the biosyntheses of succinate dehydrogenase, monoamine oxidase, sarcosine dehydrogenase, and an unknown flavoprotein were observed.

INTRODUCTION: The effect of riboflavin deficiency on the lipid composition and biochemical properties of hepatic mitochondria have been described recently (1). It was shown that protein patterns and the reduced-minus-oxidized difference spectra are distinctly different by the time succinate dehydrogenase activity is approximately 25% that of normal.

In the present work, it is shown that the mitochondrial protein patterns and the reduced-minus-oxidized spectra of mitochondria from deficient rats given an IP injection of riboflavin approach normalcy, and the enhancement in succinate dehydrogenase activity is dependent on both cytoplasmic and mitochondrial protein synthesis. When the deficient rats are given [^{14}C]riboflavin, four principal radioactive proteins are located on SDS-polyacrylamide gels. These findings further indicate that this particular system offers unique possibilities for studying mitochondrial biogenesis in a mammalian system.

MATERIALS AND METHODS: Rats weighing 45 to 55 g were fed a riboflavin-deficient diet (Teklad Mills, Madison, WI) for 35 to 45 days; a control group was fed ad libitum the same diet supplemented with 25 mg riboflavin/kg. Ani-

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mals were housed and hepatic mitochondria isolated as described elsewhere (1). Riboflavin (32 μg) was injected IP as unlabeled or ^{14}C-compound (11.9 μCi) in physiological saline (0.35 ml). Protein was determined by the procedure of Lowry et al. (2), with bovine serum albumin as standard.

Brevibacterium ammoniagenes (ATCC 6872) was cultured according to Nakamura and Tanaka (3) in the presence of [U-^{14}C]guanosine (1.5 mCi/ml, Amersham/Searle, Arlington Heights, IL) for five days. The FAD was isolated via phenol extraction and DEAE ion-exchange chromatography (4); it was converted to riboflavin by acid hydrolysis and further purified by descending chromatography on Whatman #1 paper with a developing system of n-butyl alcohol:acetic acid:water (2:1:1). The specific activity of the [^{14}C]riboflavin was determined to be 140 mCi/mmol, which is approximately six-fold that available from commercial sources.

Mitochondrial proteins (275 μg) were separated on 10 x 0.6 cm polyacrylamide gels, 9.75% acrylamide and 0.3% bisacrylamide for the resolving gel, and 3% acrylamide and 0.62% bisacrylamide for the stacking gel, which contained 0.1% SDS (5). Protein was stacked at 0.5 mA and resolved at 3 mA/tube at 20°. For labeled samples, gels were frozen on solid CO_2 and sliced with a manual gel slicer (Hoefer Scientific, San Francisco, CA). The slices (~ 1 mm) were incubated overnight at 50° with 0.63 ml of NCS solubilizer (Amersham/Searle) and 0.07 ml of water (6). After addition of 10 ml of toluene containing 6.0 g of 2,5-diphenyloxazole and 0.49 g of 1,4-bis-[2-(5-phenyloxazolyl)]-benzene/liter, samples were counted in a Packard model 3375 liquid scintillation spectrometer. For unlabeled samples, the gels were fixed and stained according to Dulaney and Touster (5).

RESULTS: In the previous report (1), it was shown that the reduced-minus-oxidized spectra of hepatic mitochondria from riboflavin-deficient rats differ from the controls. As seen in Figure 1, flavin deficiency causes enhanced absorption of the cytochrome (a_3 + a)γ band and a hypsochromic shift of approximately 2 nm of the γ band of cytochrome c_1 and the α bands of cytochromes (c + c_1). When mitochondria were obtained from rats sacrificed at 0, 3, and 24 hr after an IP injection of riboflavin, results depicted in Figure 2 were obtained. There is a precipitous decrease in the maximum at 443 nm by 3 hr and a partial reversal after 24 hr. Concomitantly, there is an increase in the broad maximum from ~ 470 to 500 nm. Generally, spectra for the 3 and 0 hr groups are almost identical above ~ 500 nm; after 24 hr, there are still subtle changes below 500 nm, but the spectrum approximates that of the ad libitum-fed controls above this wavelength.

In riboflavin-deficient rats, succinate dehydrogenase activity is greatly diminished when measured by the dye-coupled assay with phenazine methosulfate and 2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium. Kim

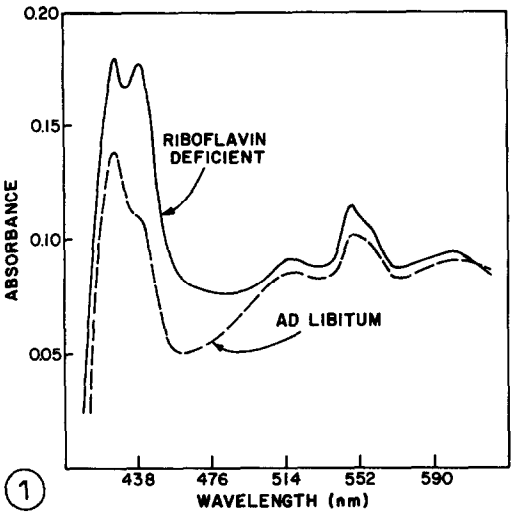

Figure 1: Reduced-minus-oxidized difference spectra of mitochondria.

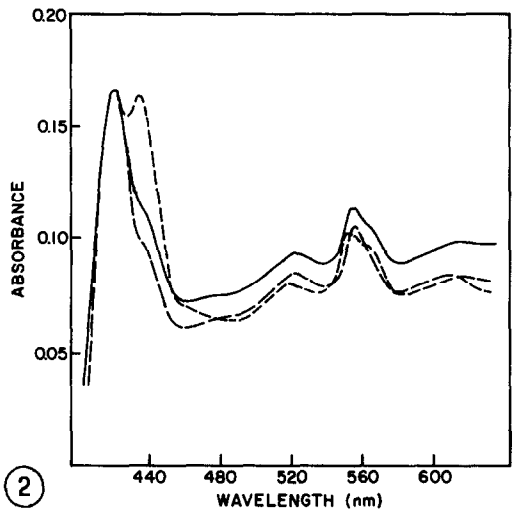

Figure 2: Reduced-minus-oxidized difference spectra of mitochondria at 0 (----), 3 (— —), and 24 (——) hr after injection of riboflavin.

and Lambooy (7) have shown that this activity is immediately enhanced during realimentation with riboflavin. As shown in Figure 3, activity of succinate dehydrogenase increases linearly up to 5 hr after IP injection of riboflavin to deficient animals. That this enhanced activity is dependent on both cytoplasmic and mitochondrial protein biosynthesis is also indicated, since cycloheximide and puromycin both inhibit. Control experiments conducted with these protein-synthesis inhibitors established that the level of cycloheximide (15 mg/kg body weight) used causes greater than 93% and puromycin (175 mg/kg body weight) greater than 60% inhibition of [^{14}C]leucine incorporation into mitochondrial proteins.

The effects of recovery of the mitochondrial proteins of riboflavin-deficient rats injected with riboflavin are depicted in Figure 4. The pattern is acutely altered in bands 1, 5, 7, and 11, with major changes already apparent by 3 hr. There is also an increasing difference between the amounts of 11 relative to 13 and a gradual increase in 10, which is almost absent in the deficient rats. Band 5 has been assigned to the 70,000 MW subunit of

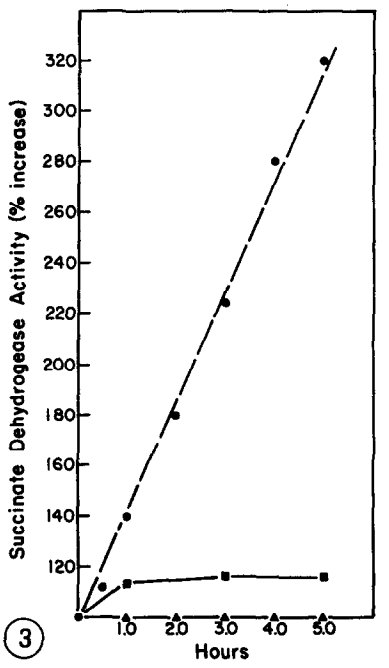

Figure 3: Changes in mitochondrial succinate dehydrogenase following injection of riboflavin to deficient rats (●) and those treated with cycloheximide (■) or puromycin (▲).

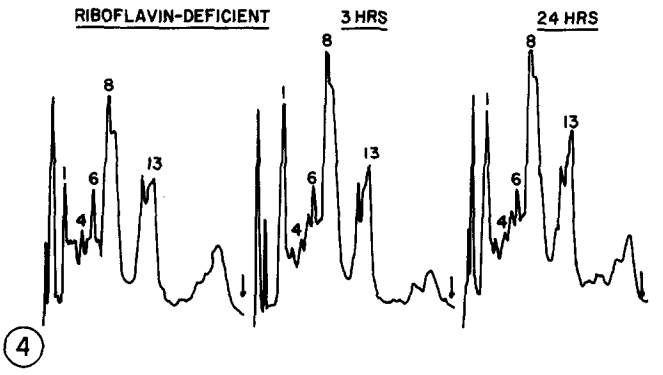

Figure 4: SDS-polyacrylamide gels of mitochondrial proteins at periods after injection of riboflavin to deficient rats.

succinate dehydrogenase reported by Hanstein et al. (8). This particular subunit has the covalently attached flavin, shown to be an N(3)-histidylFAD (9), and would be expected to incorporate [^{14}C]flavin during its biosynthesis. Indeed, this is the case, as shown in Figure 5, where the radioactive flavoprotein (indicated as II) corresponds at a MW of 70,000 to band 5 (see Figure 4). Flavoproteins III and IV are assigned to monoamine oxidase (10) and sarcosine dehydrogenase (11), respectively. The identity of I, with MW ~ 91,000, is not known, although this species was also noted by Sato et al. (12), who independently reported on the covalently bound flavin in rat liver mitochondria while the present work was in progress.

DISCUSSION: The electron microscopic studies of Tandler et al. (13) and Reith (14) have shown that enlarged mitochondria produced in rat hepatocytes

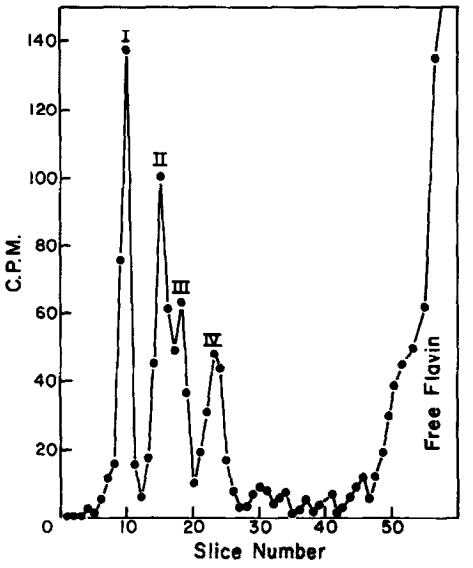

Figure 5: Incorporation of [^{14}C]riboflavin into the covalently bound flavoproteins of mitochondria. A daily injection was given over a period of four days, and the rats were sacrificed on the fifth day.

during riboflavin deficiency regress in size after three days of refeeding riboflavin and that this is preceded by the formation of structural partitions within mitochondria. Little had been done to characterize the biochemical changes that occur during this recovery process, and, until recently (1), there was not even much information on the properties of mitochondria from deficient animals. These facts suggested that this system may be suitable for studying mitochondrial biogenesis in rats during recovery on riboflavin, since the biochemical properties are distinctly different in deficient and normal states, recovery proceeds within a reasonable span of time, and changes are easily and reliably monitored.

The biosynthesis of succinate dehydrogenase and, additionally, monoamine oxidase and sarcosine dehydrogenase, as has been demonstrated in this work, ultimately leads to membrane-bound activities. It is of interest that both cytoplasmic and mitochondrial systems appear to be prerequisite to for-

mation of at least one of these, i.e. succinate dehydrogenase. Additionally, the same approach can be applied to monoamine oxidase and sarcosine dehydrogenase. Cytochrome c oxidase has been shown to be composed of seven subunits; one, with MW 23,000 (± 7%), is apparently an essential component in the functional enzyme (15). The gradual decrease in band 11, with MW 29,000 (± 8%), correlates generally with the decrease in the maximum at 443 nm assigned to the γ band of cytochrome (a_3 + a) in the reduced-minus-oxidized difference spectrum. Hence, it may be that 11 is a component of cytochrome c oxidase, but a conclusion cannot unequivocally derive from the present data. A definite answer to this, the identity of covalent flavoprotein I, and questions relating to the particular sequence and localization of biosynthetic reactions involved in the biosynthesis of flavoproteins are points for future research.

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