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Rat Liver Mitochondrial F_1-ATPase, an FAD Containing Ferroprotein

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F_1-ATPase isolated from rat liver mitochondria has been found to contain approximately 1 mole of FAD and 6 g atoms of nonheme iron per mole of enzyme.

It has been recently shown in our laboratory (11) that F_1-ATPase isolated from rat liver mitochondria may be present in two forms which are interconvertible through a redox reaction. The oxidized form revealed a much lower ATP hydrolyzing activity, together with a higher sensitivity to its stimulation by bicarbonate than the corresponding reduced form of the enzyme. The reduction of ATPase was easily achieved with dithionite and the reversion of the process was also readily accomplished with dichloroprenolindophenol.

In a search for possible components of the F_1-ATPase which might be implied in redox reactions, the presence of FAD has been demonstrated. Assuming a molecular weight for F_1-ATPase of about 360,000 the flavin to protein molar ratio was found to be approximately equal to 1. Nonheme iron was also detected and a value of 6 g atoms per mole of enzyme was obtained.

Materials and Methods

Mitochondria were obtained from male Wistar rats by the method of HOGEBOOM (8); protein determination was carried out following the technique of LOWRY *et al.* (12) using bovine serum albumin as standard. The standard values were corrected for inner membrane mitochondrial proteins by the Kjeldahl method. Purified F_1-ATPase was obtained as described by LAMBETH and LARDY (10). ATPase activity was determined essentially as described by PULLMAN *et al.* (14) in the absence of an ATP generating system, measuring the P_i liberated according to

the method of FISKE and SUBBAROW (5). The determination of succinate dehydrogenase activity was carried out as described in the literature (13). SDS gel electrophoresis was performed according to the method of FAIRBANKS *et al.* (3). Thin layer chromatography of FAD was carried out as described by FAZEKAS and KOKAI (4) using as solvent 5 % $Na_2PO_4H.2H_2O$ in distilled water. The quantitative determination of FAD was performed as described by BURCH (2). The extraction of flavins from ATPase was carried out either with trichlororacetic acid (2) or with boiling water (15). Nonheme iron was determined by atomic absorption with a Perkin-Elmer instrument Model 305 B; the wavelength was set at 248.3 nm and the other operating parameters were also as recommended in the instruction manual. For the quantitative determination of iron approximately 2 mg of enzyme in 2 ml of the medium described by LAMBETH and LARDY (10) to redissolve the enzyme after its precipitation with $(NH_4)_2SO_4$ were boiled for 5 minutes, centrifuged and the pellet reextracted again with 1 ml of the same medium, and the two supernatants pooled. The pellet was again reextracted with 1 ml of 10 % trichloroacetic acid for 15 minutes at room temperature and measured independently. 90 % of the iron was extracted by boiling in the medium and only 10 % of the total was found in the trichloroacetic acid extract. Blanks with all the reagents used were prepared for each determination. Labile sulfide was determined as described by KING and MORRIS (9).

Results and Discussion

Purified F_1-ATPase gave an electrophoretic pattern which coincided with that given in the literature (10). In agreement with LAMBETH and LARDY (10) the hydrolyzing activity of purified F_1-ATPase was oligomycin insensitive and at the same time exhibited sensitivity to bicarbonate stimulation. Specific activity was equal to 90 μmoles of ATP hydrolyzed $\times$ min^{-1} $\times$ mg^{-1} protein.

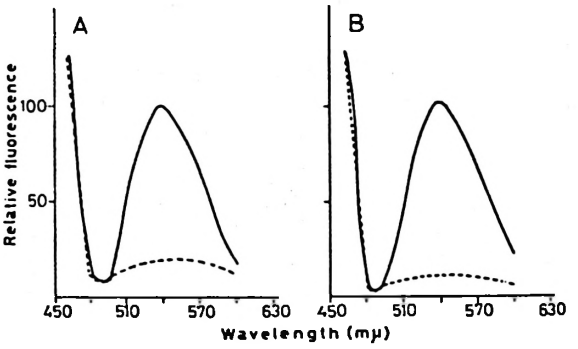

Fig. 1. *Fluorescence spectra of purified F_1-ATPase (A) and of the extracted flavins (B).* Excitation light, 450 nm. Without (———) and with added dithionite (— — —).

Figure 1 shows the fluorescence spectra of purified F_1-ATPase and of extracts prepared from the enzyme following the method described by BURCH (2) as suitable for the extraction of non-covalently bound flavins. On excitation with monochromatic light of 450 nm wavelength a maximum of light emission at 535 nm wavelength was observed. In both cases the 535 nm emission peak practically disappeared after the addition of dithionite. These data strongly suggest that a flavin was associated with the enzyme.

In order to ascertain whether the extracted flavin was FAD or FMN, two tests were preformed: *a*) TLC chromatography (4) of the aqueous extract (15) obtained from purified F_1-ATPase; it was observed that the Rf of the fluorescence spot coincided with that of an authentic sample of FAD. *b*) The extract was subjected to mild acid hydrolysis as indicated by BURCH (2); it was also seen that an enhancement of fluorescence, read at pH 6.8, took place, indicating a possible conversion of FAD to FMN.

The FAD content of F_1-ATPase was 1.95 $\pm$ 0.21 μg per mg of protein; assuming a MW for F_1-ATPase of 360,000

the flavin to protein molar ratio would be approximately 1. No activity of succinate dehydrogenase was detected thus excluding the possibility of contamination with this enzyme. The amount of nonheme iron was 0.929 ± 0.035 μg per mg of enzyme, which represents an approximate value of 6 g atoms of iron per mole of enzyme. The values of flavin and iron represented the average of determinations carried out on 10 independent F_1-ATPase preparations. The presence of labile sulfide was not detected. HATEFI *et al.* (6, 7) have reported that about 1 nmole of flavin and 3-4 μg atoms of Fe per mg protein were present in what they term complex V, obtained from beef heart mitochondria. According to these authors complex V appears to contain together with the F_1-ATPase the oligomycin sensitivity conferring protein (OSCP), the dicyclohexylcarbodiimide (DCCD) binding protein as well as a few additional polypeptides. The presence of FAD as well as iron in purified F_1-ATPase does not seem to have been reported until the present work.

FAD and iron might very well be implicated in the interconversion of the two forms of the enzyme by a redox reaction. This finding may also throw some light on our understanding of the mechanism of energy transduction. The shortening of the ionic radius of the atoms of iron when passing from Fe^{++} to Fe^{+++}, together with the different conformations of the reduced and oxidized forms of the flavin might very easily provoke extensive conformational changes of the protein. The presence of iron and FAD in ATPase seems to offer a new molecular basis to the alternating site model for the conformational coupling to ATP synthesis as recently suggested by BOYER (1).

Resumen

Se ha encontrado que la F_1-ATPasa aisalda de mitocondrias de hígado de rata contiene aproximadamente 1 mol de FAD y 6 átomos gramo de hierro no hemo por mol de enzima.

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