Changes in activity and F_1 content of mitochondrial H^+-ATPase in regenerating rat liver

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Submitochondrial particles prepared from rat liver during hepatic regeneration exhibit a depressed ATPase activity which is correlated with a decrease in F_1 subunit content as shown by SDS-PAGE. Use of an antibody directed against the F_1 portion of the H^+-ATPase complex demonstrated that there is a definite decrease in the amount of β-subunit of F_1 in both submitochondrial particles and mitochondria from rat liver 24 h after partial hepatectomy

H^+-ATP synthase *Liver regeneration* *Mitochondria*

1. INTRODUCTION

The subunit composition of mammalian F_0F_1-ATPase is still not completely characterized. It is established that there are 5 cytosolically synthesized subunits of the catalytic F_1 sector and an ATPase inhibitor protein [1,2]. The number of subunits of the F_0 membrane moiety, whilst well defined as being 3 in prokaryotic systems [3,4], remains unclear in mammalian mitochondria [5,6] and may well amount to 7 [7]. This study concerns the F_1 sector of the mitochondrial H^+-ATPase complex in early regenerating rat liver. Rat liver regeneration involves marked re-organisation of cell and tissue mass [8,9] characterised by a phase of retro-differentiation followed by re-differentiation.

Kinetic analysis of ATPase activity of submitochondrial particles prepared from rat liver showed a substantial decrease in ATPase activity, with maximal depression occurring 24 h after a partial (70%) hepatectomy conducted under a general ether-oxygen anaesthesia. Furthermore electrophoresis and immunodecoration indicated a decrease in F_1 content in both mitochondria and submitochondrial particles at 24 h after partial hepatectomy.

A preliminary account of these observations has been presented [10].

2. MATERIALS AND METHODS

2.1. *Materials*

Phospho*enol*pyruvate, pyruvate kinase, lactate dehydrogenase, β-nicotinamide adenine dinucleotide, reduced form and adenosine 5-triphosphate were obtained from Boehringer (Mannheim). All other chemicals were of the highest purity grade available.

2.2. *Partial hepatectomy*

Male Wistar rats (300 g) were anaesthetized using ether-oxygen [11] and the median and left lateral lobes of the liver excised (corresponding to 65–70% net weight of the whole liver). The animals were maintained on a standard diet for 24 h (12 h light, 12 h dark cycle) then killed by decapitation and mitochondria isolated as in [12]. All operations were carried out under conditions that were as sterile as possible. Sham-operated rats were subjected to the same treatment without excision of the liver.

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2.3. *Preparation of submitochondrial particles*

Inside-out submitochondrial particles were obtained by exposure of rat liver mitochondria that had been stored frozen at −70°C for 24 h to ultrasonic energy in the presence of EDTA (4 mM) at pH 8.5 [13,14] (ESMP).

2.4. *Isolation of F_1*

F_1 was isolated as in [15] except that the gel filtration and lyophilization steps were omitted.

2.5. *Determination of ATPase activity*

ATPase activity was determined in the presence of added pyruvate kinase, phospho*enol*pyruvate and lactate dehydrogenase by following NADH oxidation spectrophotometrically at 340 nm in a thermostatically controlled reaction cell at 30°C. The reaction mixture consisted of 250 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.5, 0.025 mM NADH, 0.5 μg rotenone, 1 mM phospho*enol*pyruvate, 2.5 units lactate dehydrogenase, 2 units pyruvate kinase and 20–30 μg submitochondrial protein or 2–3 μg soluble F_1 protein in a final volume of 1 ml. The reaction was started by the addition of ATP at the concentrations reported in the figure legends. Protein was determined by the Lowry method.

2.6. *Gel electrophoresis and immunodecoration*

12–20% polyacrylamide gels containing SDS and 3.2 mM urea were prepared essentially as in [16]. Western type blotting to nitrocellulose sheets and subsequent immunodecoration was carried out as in [17] using an antibody directed against the bovine ATPase F_1. Visualization of the reactive antibody-antigen complexes was effected using goat anti-rabbit-antibody conjugated to horseradish peroxidase.

3. RESULTS

Determination of the ATP hydrolase activity of ESMP from control and hepatectomized rats was routinely obtained from saturation curves plotted according to Lineweaver and Burk. It was consistently noted that the ATPase activity of ESMP changed markedly in the course of liver regeneration (table 1). A significant decrease of the V_{max} (75%) with respect to sham-operated liver was observed after 24 h regeneration. This depression, however, rapidly disappeared with time such that after 48 h ATPase activity returned to control levels. Independent controls showed that there was no change in ATPase activity and F_1 content in ESMP from control rats killed at various intervals from 2 to 48 h after exposure to oxygen-ether anaesthesia. There is ample evidence that the use of this general anaesthesia produces no long-term changes (longer than the duration of the anaesthesia) in the phosphate potential either in control or early regenerating rat liver [11].

Table 1

Changes in the hydrolase activity of mitochondrial H^+-ATPase during rat liver regeneration

Time of regeneration (h)	V_{max} ($\mu mol \cdot min^{-1} \cdot$ mg protein^{-1})	% decrease	K_m (mM)
0	1.13	–	0.17
12	0.46	40	0.20
24	0.28	75	0.19
48	0.98	13	0.18
72	1.30	0	0.19

ATPase activity of ESMP prepared from control rat liver (sham-operated animals) and rat liver during regeneration was measured as μmol phospho*enol*pyruvate hydrolysed as described in section 2. 20–30 μg ESMP protein were incubated in 1 ml of the mixture described in section 2, final pH 7.5, temperature 30°C

Consequently changes occurring following 24 h of regeneration were studied.

As shown in table 2 there is indeed a significant reduction in the ATPase activity of ESMP from regenerating rat liver with respect to control. To exclude the possibility that the measured lower ATPase activity was not due to an increase in non-specific protein concentration during regeneration, ATPase activity was expressed on a per heme $a + a_3$ basis. The same degree of depression of activity was observed. It should be noted that controls showed that there was no change in cytochrome oxidase and other cytochromes content in regenerating (24 h) compared to control rat liver.

An enhanced lability of the F_1 moiety during regeneration may result in facilitated loss of F_1 subunits during the preparation of submitochondrial particles. Use of an antibody directed against

bovine heart F_1, cross reacted predominantly with the rat liver β-subunit. As shown in fig.1, electrophoresis followed by electrotransfer to nitrocellulose sheets and subsequent immunodecoration showed a decrease in the quantity of β-subunit present in intact mitochondria from regenerating (24 h) with respect to control (sham-operated) rat liver.

The presence of magnesium and ATP stabilizes the F_1–F_0 interaction in intact H^+-ATPase [13]. As shown in table 2, whilst the relative ATPase activity of submitochondrial particles prepared in the presence of ATP and Mg was slightly higher than that of ESMP [18], the same extent of depression of the ATPase activity was found in Mg-ATP submitochondrial particles prepared from regenerating rat liver, with respect to controls.

It should be noted that loss of ATPase activity occurs during ESMP preparation. In preparations of ESMP from both control and regenerating rat liver a total ATPase activity, corresponding to 15% of the final ATPase activity calculated in ESMP, was found in the supernatant following sonication. The addition of Mg-ATP, by enhancing F_1–F_0 interactions, reduced the total ATPase activity recovered in the supernatant to approx. 3% in preparations from both control and

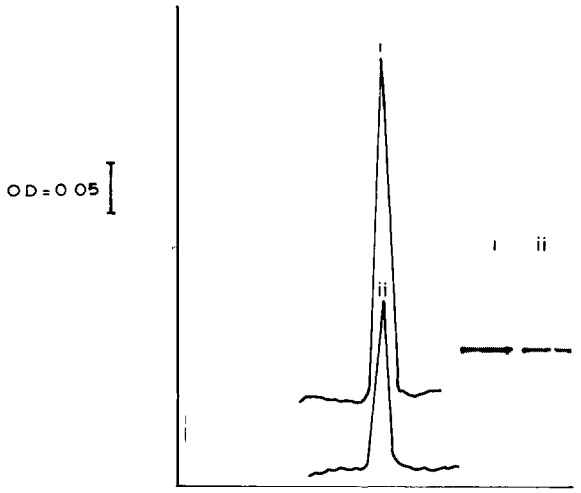

Fig.1. Immunoblots and densitometric traces of immunoblots of mitochondria from control rat liver and rat liver following 24 h regeneration. SDS-PAGE on 12–20% polyacrylamide gels containing 3.2 M urea and subsequent electrotransfer onto nitrocellulose sheets were carried out as described in section 2. Photographs of immunoblots were developed onto translucent sheets which were scanned using a Gelman DCD-16 densitometer. (i) Mitochondria from control rat liver (2.5 μg applied to gel). (ii) Mitochondria from regenerating rat liver (2.5 μg applied to gel).

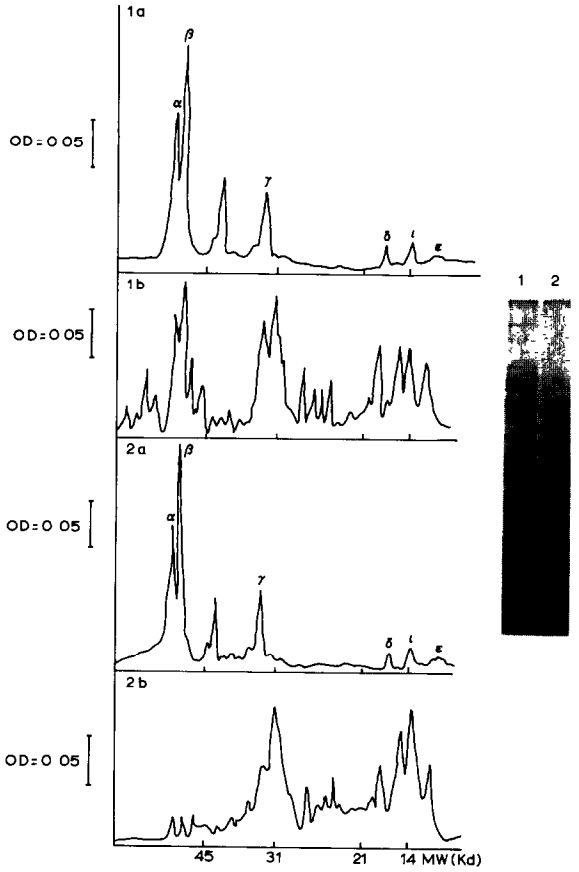

Fig.2. Electrophotograms and densitometric traces of submitochondrial particles prepared in the presence of EDTA from control (sham-operated) rat liver and rat liver following 24 h regeneration and densitometric trace of soluble F_1 isolated from control and regenerating (24 h) rat liver. SDS-PAGE was carried out as described in the legend to fig.1 and in section 2. (1) Submitochondrial particles (ESMP) from control rat liver (100 μg applied to gel). (2) Submitochondrial particles (ESMP) from regenerating (24 h) rat liver (100 μg applied to gel). (1a) Densitometric scan of soluble F_1 isolated from control rat liver (20 μg applied to gel). (1b) Densitometric scan of lane 1 (ESMP from control rat liver). (2a) Densitometric scan of soluble F_1 isolated from regenerating rat liver (20 μg applied to gel). (2b) Densitometric scan of lane 2 (ESMP from regenerating rat liver).

Table 2

(A) ATPase activity of submitochondrial particles and F_1 prepared from control (sham-operated) and regenerating (24 h) rat liver. (B) ATPase activity of supernatants from submitochondrial particle preparation from control (sham-operated) and regenerating (24 h) rat liver

(A) Sample	V_{max} (μmol·min^{-1}· mg protein^{-1})	K_m (mM ATP)	V_{max} (μmol·min^{-1}· nmol hemes $a+a_3^{-1}$)
Control ESMP	1.32 ($\pm$ 0.12) n = 6	0.17 ($\pm$ 0.01) n = 6	1.49
Regenerating ESMP	0.3 ($\pm$ 0.06) n = 6	0.17 ($\pm$ 0.02) n = 6	0.35
Mg·ATP·ESMP from control rat liver	1.6	0.2	–
Mg·ATP·ESMP from regenerating rat liver	0.53	0.21	–
Sephadex SMP from control rat liver	2.0	0.19	
Sephadex SMP from regenerating rat liver	0.7	0.19	
ESMP from regenerating rat liver; plus inhibitors of proteolysis	0.32	0.18	
[a]F_1 from control rat liver	5.0	0.2	
[a]F_1 from regenerating rat liver	6.2	0.2	

regenerating rat liver. ATPase activity in the supernatant obtained from the preparation of both ESMP and Mg-ATP submitochondrial particles from regenerating rat liver amounted to 65–70% less than the activity in the supernatant from the preparation of particles from control rat liver.

An enhanced concentration and/or a tighter association of the inhibitor protein [19] may contribute to the depression of ATPase activity seen in submitochondrial particles from regenerating rat liver. ESMP particles were treated to remove the inhibitor protein [20]. As shown in table 2, whilst there was an expected increase in ATPase activity in particles from both control and regenerating rat liver there remained a relative decrease in the latter with respect to the control.

Gel electrophoresis of ESMP from control and regenerating rat liver (fig.2) showed that there is a clear decrease of the bands corresponding to the α, β and γ subunits of F_1 (cf. densitometric trace of PAGE of F_1 from control rat liver mitochondria) in ESMP from regenerating rat liver compared to control.

It should be noted that PAGE of ESMP does not provide a high resolution of the low-M_r proteins. Consequently, assignment of the δ and ϵ subunits is not possible here due to the presence of other proteins having similar R_f values.

In separate experiments mitochondria and submitochondrial particles were prepared from regenerating rat liver in the presence of the protease inhibitors phenylmethylsulphonyl fluoride (PMSF), *o*-phenanthroline and EDTA. The same degree of depression of ATPase activity (table 2)

(B) Sample	Total activity recovered in the supernatants (μmol·min^{-1})	Total activity of SMP (μmol·min^{-1})	Activity in the supernatant as a percentage of that found in submito-chondrial particles
Supernatant from ESMP preparation from control rat liver	0.57	3.9	14.6%
Supernatant from ESMP preparation from regenerating rat liver	0.13	0.8	16.2%
Supernatant from Mg·ATP·SMP preparation from control rat liver	0.12	6.12	2.0%
Supernatant from Mg·ATP·SMP preparation from regenerating rat liver	0.055	1.74	3.1%

[a] The relatively low activities of these enzyme proteins may be ascribed to the presence of the inhibitor protein since Sephadex chromatography and lyophilization steps were omitted during their preparation

(A) Heme $a+a_3$ content was measured spectrophotometrically as in [23]. ESMP from control rat liver possessed 0.88 ($\pm$ 0.02, n = 6) nmol hemes $a+a_3$·mg protein^{-1} and ESMP from rat liver after 24 h of regeneration 0.86 ($\pm$ 0.02, n = 6) nmol hemes $a+a_3$·mg protein^{-1}. 20–30 μg submitochondrial particles or 5 μg F_1 were incubated in 1 ml of the reaction mixture as described in section 2

(B) Approx. 10 mg mitochondrial proteins were sonicated to prepare submitochondrial particles. The total recovered particle proteins in the various preparations amounted to 28–36% of mitochondrial protein, whilst the amount of proteins recovered in the supernatants amounted to 36–40% of mitochondrial protein. The ATPase activity of both the particles and supernatant was measured at saturating concentration of ATP

and an equal diminution in α and β subunit content (not shown) were observed, as in ESMP from regenerating rat liver prepared in the absence of inhibitors of proteolysis, compared to controls.

Isolation of soluble F_1 from mitochondria produced an enzyme preparation from regenerating rat liver which exhibited the same polypeptide composition (fig.2) and the same specific activity and K_m for ATP (table 2) as the enzyme obtained from control rat liver. In particular, it may be noted that the content of the inhibitor protein in the preparation from regenerating rat liver was apparently the same as in F_1 prepared from control rat liver. The yield of F_1 (mean of 4 experiments) obtained from regenerating rat liver was half of that from the corresponding controls.

4. DISCUSSION

Our results reveal a decrease in ATPase activity in submitochondrial particles which is related to a diminution in F_1 content. This decreased F_1 content is not due to trivial loss of F_1 during ESMP production. In fact, immunoblotting showed a decreased content of the β-subunit of the ATPase in intact mitochondria from regenerating rat liver with respect to control. Isolation of F_1 from regenerating rat liver mitochondria gave a reduced yield of the enzyme and no apparent change in subunit stoichiometry could be detected compared to F_1 from control rat liver. Furthermore no enhanced ATPase activity was observed in the supernatants following sonication in the preparation of ESMP from regenerating rat liver compared to control.

Comparison of the electrophoretic pattern of ESMP (fig.2) shows a decrease of the α, β and γ subunits of F_1 in ESMP from regenerating rat liver compared to controls. PAGE of ESMP does not allow assessment of the δ and ϵ subunits. It can, however, be noted that there was a reduced yield of F_1 isolated from regenerating rat liver mitochondria compared with controls with no apparent change in the relative polypeptide composition of the F_1 from regenerating rat liver with respect to controls. The band appearing below the β subunit in the densitometric traces of soluble F_1 may correspond to the extra band noted in the preparation from [15] and may be an aggregate or cross-linked product of the inhibitor protein.

An enhanced degree of proteolysis associated with the early phase of retro-differentiation would explain the observed decrease in F_1 content. However no decrease in other inner membrane associated proteins, for example cytochrome oxidase and cytochrome *c* was observed. The possibility of enhanced proteolysis during ESMP production from regenerating rat liver was ruled out by the inclusion of the proteolysis inhibitors (PMSF, *o*-phenanthroline and EDTA).

The transient decrease in F_1 content occurs at the initial necrotic retro-differential phase of regeneration which is associated with changes in lipid and protein metabolism [21] and a dramatic increase in DNA replication which peaks 24 h following partial hepatectomy [8]. It is tempting to speculate that there are changes in either the synthesis and processing, or uptake into mitochondria of F_1-subunits or, at least, subunits critical for the assembly of an integral F_1 moiety during early regeneration.

We are currently investigating the effect of early rat liver regeneration of the F_0 membrane sector of the H^+-ATPase.

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