Regulation of ATP hydrolase activity of the F_0-F_1 complex of rat-liver mitochondria during early hepatic regeneration

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Submitochondrial particles prepared from rat liver in the early phase of hepatic regeneration possess a reduced F_1 content with respect to F_0 in intact F_0F_1-H^+-ATPase complexes. Analysis of ATP hydrolysis showed a significant difference in both ESMP and isolated F_1 with regard to the higher affinity K_m values ($K_{m,1}$) obtained from Eadie-Hofstee plots. Both ESMP and F_1 from regenerating rat liver showed much lower apparent $K_{m,1}$ values (0.04 and 0.03 mM, respectively) than the corresponding controls (0.08 mM for both ESMP and F_1). Data presented here show that the residual F_1 moieties have an altered kinetic pattern with regard to the competitive inhibitor adenosine 5′-[β,γ-imido]triphospate (K_1 ESMP from regenerating rat liver = 0.67 μM, K_1 ESMP from control rat liver = 2.03 μM). This difference in affinity for [β,γ-imido]-ATP is also seen in isolated F_1 (K_1 regenerating rat liver = 0.04 μM, K_1 control rat liver = 0.22 μM). These data indicate that during the disruptive retrodifferential phase of hepatic regeneration, changes at the level of surviving F_1 sectors of the F_0-F_1 ATPase may play a physiological role in preventing ATP hydrolysis in vivo in the brief period of low $\Delta\mu H^+$, induced by the presence of non-F_1-associated F_0 proton-conducting pathways.

F_1F_0-H^+-ATP synthase *Liver regeneration* *Mitochondria*

1. INTRODUCTION

During the early retrodifferential premitotic phase of hepatocyte regeneration induced by partial hepatectomy, the mitochondria of such cells are uncoupled with regard to oxidative phosphorylation due to the absence of a large proportion of the F_1 catalytic sectors of the F_0F_1-H^+-ATPase [1], and the consequent presence of uncontrolled proton flux through non-F_1-associated F_0 moieties [2].

This transient phase is anomalous in that it not only restricts ATP generation to non-oxidative phosphorylation pathways at a period in the cell cycle when there is a large requirement for ATP for growth and repair, but also leads to the possibility of increased ATP hydrolysis by the remaining (around 30% of control) F_1 sectors of the F_0F_1-H^+-ATPase in the absence of a transmembrane $\Delta\mu H^+$.

It has been suggested that mitochondria possess two mechanisms whereby ATP hydrolysis by the ATPase may be restricted during conditions of low $\Delta\mu H^+$ [3]. The first of these is a rapid response involving changes in the strength of ADP binding, a so-called fast regulatory device; the formation of an inactive complex between ADP and the active site of the ATPase resulting from a release of phosphate without subsequent release of ADP [4].

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Abbreviations: F_0, membrane integral sector of mitochondrial H^+-ATPase; F_1, catalytic part of mitochondrial H^+-ATPase; ESMP, submitochondrial particles prepared in the presence of EDTA; [β,γ-imido]ATP, adenosine 5′-[β,γ-imido]triphosphate. Enzymes: ATPase (EC 3.6.1.3); pyruvate kinase (EC 2.7.1.40); lactate dehydrogenase (EC 1.1.1.27)

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This temporary inhibition is readily reversed by either the rebinding of phosphate or the restoration of $\Delta\mu H^+$ [5]. The second mechanism invoked for long periods of low or absent $\Delta\mu H^+$ involves an enhanced inhibition by the ATPase inhibitor protein [6], which suppresses ATP hydrolysis by increasing its interaction with the ATPase at some stage in the hydrolytic pathway. In this case also the regulatory effect of the inhibitor protein is controlled by the degree of energisation of the mitochondrial membranes [5].

In certain types of hepatoma cells there is a relative increase in the quantity of the inhibitor protein associated with the mitochondrial ATPase [7], even though there is generally a respectable $\Delta\mu H^+$ present. However, as previously demonstrated there is no enhancement in inhibitor protein activity in mitochondrial membranes during early hepatic regeneration [1].

Results reported here show that there are substantial differences in the K_i values for [β,γ-imido]ATP and the 'high affinity' constants ($K_{m,1}$) for ATP hydrolysis in both submitochondrial particles (ESMP) and soluble F_1 from regenerating rat liver compared to controls, suggesting that the absence of a $\Delta\mu H^+$ in mitochondria during early hepatic regeneration is associated with a response at the level of F_1 which serves to control ATP hydrolysis temporarily.

2. MATERIALS AND METHODS

2.1. *Materials*

Phospho*enol*pyruvate, pyruvate kinase, lactate dehydrogenase reduced form, adenosine 5'-triphosphate and [β,γ-imido]ATP were obtained from Boehringer (Mannheim). All other chemicals were of the highest purity grade available.

2.2. *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 0.25 M sucrose, 4.8 mM $MgCl_2$, 65 mM Tris-bicarbonate (pH 8.2), or 20 mM Tris-HCl (pH 7.2), 0.5 μg rotenone (only when ESMP were involved), 0.25 mM phospho*enol*pyruvate, 2 units lactate dehydrogenase, 2 units pyruvate kinase and either 20–30 μg ESMP or 2–3 μg isolated F_1, in a final volume of 1 ml, essentially as described in [1]. The reaction was started by the addition of ATP in the

Table 1

Apparent kinetic constants calculated from reciprocal plots of ATP hydrolysis by ESMP and isolated F_1 in the presence and absence of the competitive inhibitor [β,γ-imido]ATP

Sample	V_{max} (mol·min^{-1}·mg^{-1} protein)	K_m (mM ATP)	K_i (μM [β,γ-imido]ATP)
Control ESMP	1.32 ± 0.12 (n = 6)	0.17± 0.01 (n = 6)	2.03 ± 0.26 (n = 5)
Regenerating ESMP	0.30 ± 0.06 (n = 6)	0.17 ± 0.02 (n = 6)	0.67 ± 0.19 (n = 5)
Control F_1	4.74 ± 1.35 (n = 4)	0.22 ± 0.03 (n = 4)	0.22 ± 0.03 (n = 3)
Regenerating F_1	5.83 ± 0.85 (n = 3)	0.23 ± 0.02 (n = 3)	0.04 ± 0.015 (n = 3)

ESMP (25 μg protein·ml^{-1}) or soluble F_1 (2 μg protein·ml^{-1}) were incubated in the reaction mixture in Tris-HCl buffer (no HCO_3^-) as described in section 2. V_{max} and apparent K_m values were calculated from Lineweaver-Burk plots, and K_i values for [β,γ-imido]ATP obtained from Dixon plots of the type shown in fig.1

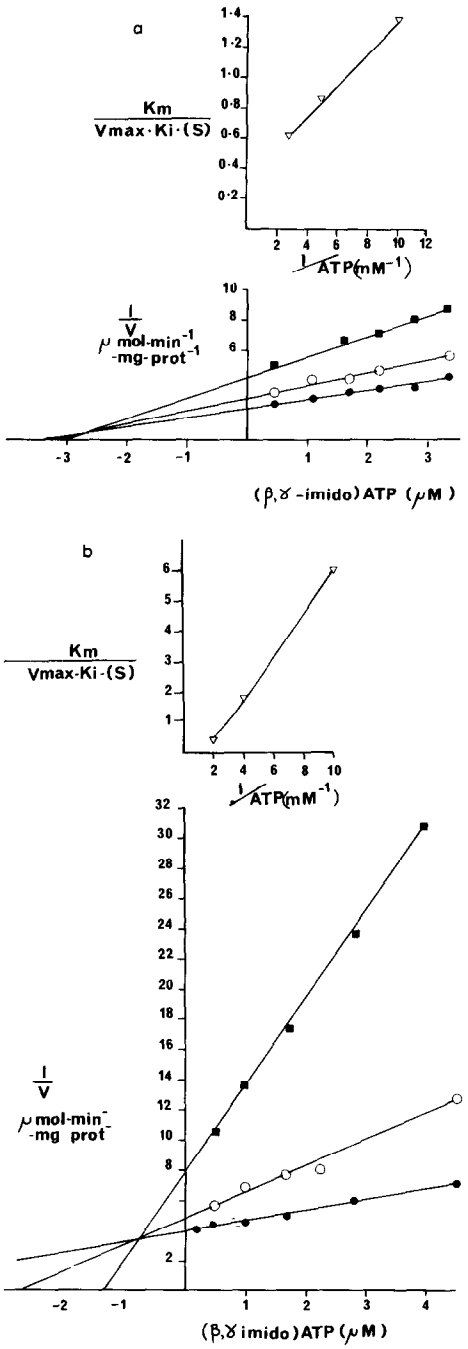

Fig.1. Dixon plots of [β,γ-imido]ATP inhibition of ATPase activity in ESMP prepared from control and regenerating rat liver. ATPase activity was measured by incubating ESMP (25 μg protein·ml^{-1}) of the reaction mixture as described in section 2 and following NADH oxidation at 340 nm at [β,γ-imido]ATP concentrations of 0.5–4 μM and the following concentrations of ATP: (■—■) 0.1 mM, (○—○) 0.2 mM and (●—●) 0.5 mM. The dissociation constant K_i calculated from this type of graph was 2.03 μM (± 0.26) for ESMP from control rat liver and 0.67 μM (± 0.19) from regenerating rat liver (see table 1). (a) ESMP from control rat liver. (b) ESMP from regenerating rat liver. The insets show plots of the slope $K_m/V_{max}\cdot K_i\cdot$[ATP] vs 1/[ATP].

concentrations reported in the figure legends. Where added, [β,γ-imido]ATP in the concentrations listed in the figure legends was allowed to incubate in the reaction mixture at 30°C for 3 min prior to the addition of ATP.

2.3. *Protein measurements*

Protein was determined by the method of Lowry et al. [8].

2.4. *General methods*

Partial hepatectomy, and the preparation of submitochondrial particles and soluble F_1, were carried out as described in [1,2].

3. RESULTS

ATPase activity in both ESMP and isolated F_1 can be represented in the form of Lineweaver-Burk plots which allow calculation of V_{max} and apparent K_m values. Such values are listed in table 1, where as previously reported [1,2] ESMP isolated during the early phase (up to 24 h) of hepatic regeneration have a diminished ATPase activity compared to controls, due as shown in [1] to a decreased quantity of F_1 associated with the membrane. As expected, although isolation of F_1 was in a lower yield (about 50–60%) from regenerating rat liver than from controls (see also [2]), there was no significant difference in either V_{max} or K_m (calculated from Lineweaver-Burk plots and shown in table 1) between the various F_1 preparations.

The addition of the non-hydrolysable competitive inhibitor [β,γ-imido]ATP to the ATPase assay experiments allowed the resulting ATPase activity of ESMP at different ATP and [β,γ-imido]ATP concentrations to be determined using Dixon plots of the type shown in fig.1, from which the dissociation constant K_i could be calculated. It is clear from fig.1 that there is a large difference between the value for the dissociation constant K_i

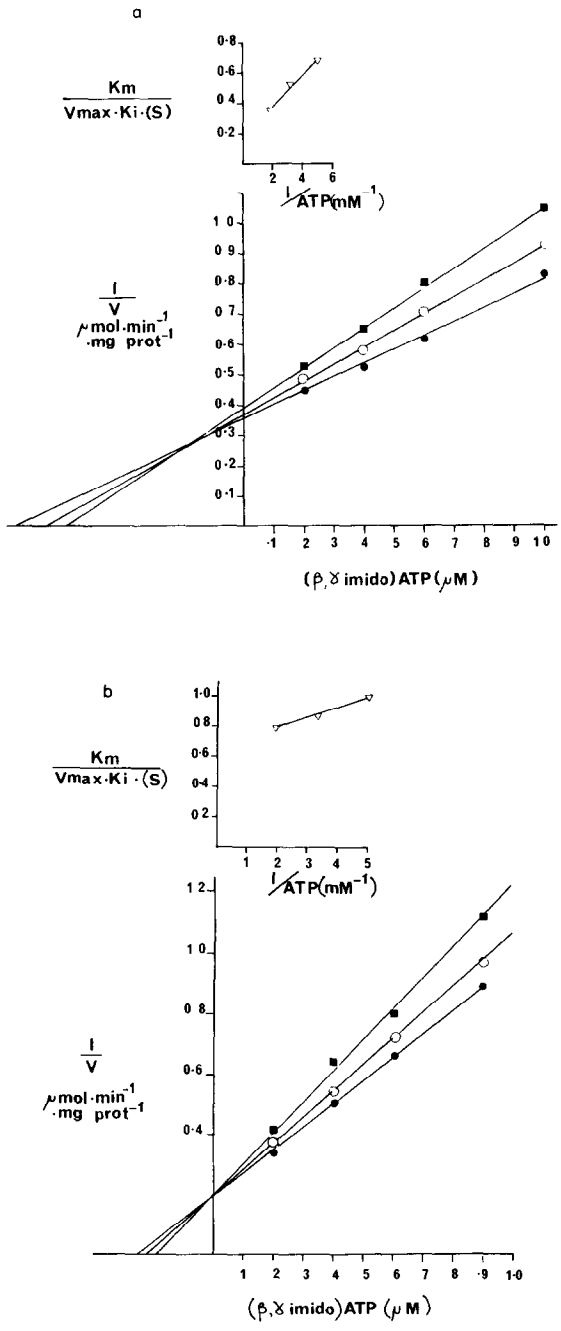

Fig.2. Dixon plots of [β,γ-imido]ATP inhibition of ATPase activity in F_1 isolated from control and regenerating rat livers. ATPase activity was measured as described in section 2 and in the legend to fig.1 in the presence of [β,γ-imido]ATP at concentrations of 0.5–4 μM. ATP concentrations were: (■—■) 0.2 mM, (○—○) 0.3 mM and (●—●) 0.5 mM. The dissociation constants K_i calculated from this type of graph were 0.22 μM (± 0.03) for F_1 from control rat liver and 0.04 μM (± 0.015) for F_1 from regenerating rat liver (see table 1). (a) F_1 isolated from control rat liver. (b) F_1 isolated from regenerating rat liver. The insets show plots of the slope, $K_m/V_{max}\cdot K_i\cdot$[ATP] vs 1/[ATP].

([β,γ-imido]ATP) obtained for ESMP from control (fig.1a) and regenerating (fig.1b) rat liver. Furthermore, these differences are also seen in Dixon plots obtained using isolated F_1 (fig.2). Table 1 includes a set of values for K_i ([β,γ-imido]ATP) for both ESMP and isolated F_1 from a range of different preparations. It can also be seen from the insets to figs 1 and 2 showing the slope ($K_m/V_{max}\cdot K_i\cdot$[ATP]) vs 1/[ATP], that the intercepts are not at ($K_m/V_{max}\cdot K_i$[ATP]) = 0 indicating that the inhibition is of a mixed competitive type. It should be noted that the intercept for fig.1b, i.e. for ESMP from regenerating rat liver, would be a negative value were the extrapolation linear. This effect, however, is artefactual since the concentration of F_1 in ESMP from regenerating rat liver is at least 30% of that in control ESMP in the reaction cuvette, hence at high ATP concentrations the curve is not linear. This effect is not observed when F_1 from regenerating rat liver alone is used (fig.2) or when higher concentrations of ESMP from regenerating rat liver are added (not shown).

Since each F_1 moiety is composed of three active sites with regard to ATP hydrolysis [9,10], Lineweaver-Burk plots do not reflect very accurately the kinetic situation with regard to the ATPase complex. Representation of the data in Eadie-Hofstee plots as shown in fig.3 permits the calculation of at least two apparent K_m values ($K_{m,1}$ and $K_{m,2}$), which are related to two of the catalytic sites on the enzyme [10]. These plots were made for both ESMP (fig.3a) and isolated F_1 (fig.3b), and the resulting V_{max} and K_m values are shown in table 2. It is clear that there is a substantial difference, seen in both ESMP and F_1, between the lower K_m values ($K_{m,1}$) which are much less in ESMP and F_1 from regenerating rat liver (0.04 and 0.03 mM, respectively) than from controls (0.08 mM for both ESMP and F_1). It should be noted that the $K_{m,1}$ values shown here are higher than those reported in [11] for bovine heart

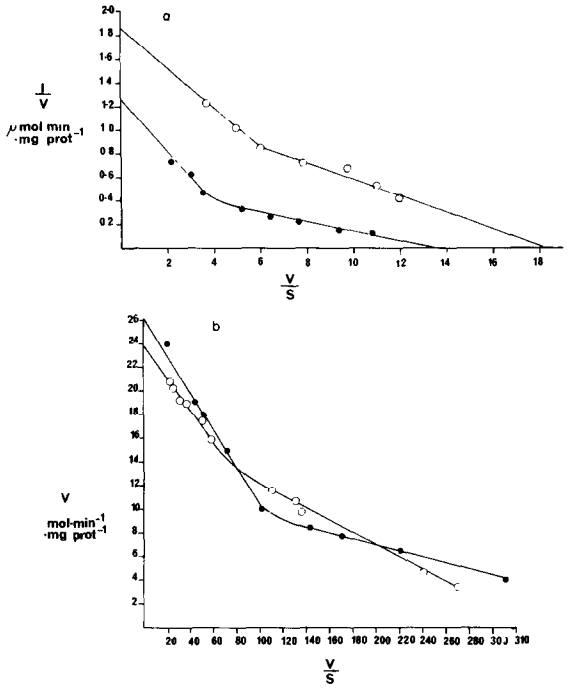

Fig.3. Eadie-Hofstee plots of the ATPase activity of ESMP and F_1 isolated from control and regenerating rat liver. ESMP (25 μg protein·ml^{-1}) or soluble F_1 (2 μg protein·ml^{-1}) were incubated in the reaction mixture in the presence of HCO_3^- as described in section 2 and in the legend to table 2. (a) ESMP from control rat liver (○—○) and ESMP from regenerating rat liver (●—●). (b) F_1 from control rat liver (○—○) and regenerating rat liver (●—●). V_{max} and K_m values calculated from these types of graphs are reported in table 2.

F_1-ATPase. This difference may be species-specific, relating to the extent of bound nucleotides, especially ADP, but in any case it does not bear upon the observed significant difference between $K_{m,1}$ values in F_1 from control and regenerating rat liver.

4. DISCUSSION

During the early retrodifferential phase of rat liver regeneration it is not at all clear why surviving hepatocytes possess a mitochondrial ATPase that consists of the normally prohibitive combination of F_0 sectors associated with a substoichiometric amount of the catalytic F_1 moiety, a situation that uncouples oxidative phosphorylation by allowing passive proton backflow into the mitochondria, hence dissipating the $\Delta\mu H^+$ which could lead to uncontrolled ATP hydrolysis by the ATPase itself. It may well be that the orchestrated synthesis, assembly and proteolytic elimination of the various subunits of this digenetically coded enzyme complex are temporarily disturbed by the changes known to occur at the level of gene expression during the first 24 h of hepatic regeneration [12]. The unnecessary hydrolysis of ATP may however be avoided at least temporarily, by a mechanism whereby a tighter binding of ADP inhibits ATP hydrolysis by residual F_1 sectors of the ATPase.

In ESMP from regenerating rat liver the presence of markedly lower K_i values for the com-

Table 2

Kinetic parameters of ATPase activity of ESMP and F_1 isolated from control and regenerating rat liver

Sample	V_1 (mol·min^{-1}·mg protein^{-1})	$K_{m,1}$ (mM ATP)	V_2 (mol·min^{-1}·mg protein^{-1})	$K_{m,2}$ (mM ATP)
Control ESMP	1.18 ± 0.13 (n = 3)	0.08 ± 0.01 (n = 3)	1.82 ± 0.08 (n = 3)	0.18 ± 0.01 (n = 3)
Regenerating ESMP	0.53 ± 0.03 (n = 3)	0.04 ± 0.01 (n = 3)	1.18 ± 0.08 (n = 3)	0.19 ± 0.03 (n = 3)
Control F_1	18.4 ± 3.95 (n = 3)	0.08 ± 0.003 (n = 3)	26.4 ± 6.33 (n = 3)	0.23 ± 0.03 (n = 3)
Regenerating F_1	10.7 ± 2.59 (n = 3)	0.03 ± 0.008 (n = 3)	23.4 ± 2.9 (n = 3)	0.19 ± 0.07 (n = 3)

ESMP (25 μg protein·ml^{-1}) or soluble F_1 (2 μg protein·ml^{-1}) were incubated in the reaction mixture in the presence of HCO_3^-, as described in section 2. V_{max} (V_1 and V_2) and apparent K_m ($K_{m,1}$ and $K_{m,2}$) values were calculated from the type of Eadie-Hofstee plot shown in fig.1

petitive inhibitor [β,γ-imido]ATP compared to ESMP from controls (table 1) shows that remaining F_1 sectors bind [β,γ-imido]ATP much more tightly than their control counterparts. That this effect is also seen in isolated F_1 confirms that it is an F_1-associated phenomenon.

The use of Eadie Hofstee plots allows a more discerning analysis of the equilibrium constants involved in ATP hydrolysis by the ATPase complex and showed that there was indeed a significant difference between the high-affinity $K_{m,1}$ values (table 2), both ESMP and F_1 from regenerating rat liver having lower $K_{m,1}$ values than from control rat liver.

An increasingly popular model for ATP hydrolysis originally proposed in [13] has also been adapted to account for [β,γ-imido]ATP binding and inhibition [14]. It is highly likely that both ADP and [β,γ-imido]ATP share an identical critical binding site on the F_1 molecule [15]. That F_1 from regenerating rat liver possesses a higher affinity for [β,γ-imido]ATP as well as a lower high-affinity $K_{m,1}$ than F_1 from control argues that F_1 from regenerating rat liver utilises a tighter binding for ADP and that this may serve as a temporary mechanism in vivo to control ATP hydrolysis by F_1 moieties which are not under the control of a $\Delta\mu H^+$. Whether this response is due to a change induced in the enzyme complex at the level of subtle subunit interactions by some agent or series of events associated with early hepatic regeneration, or is a normal consequence of the absence of a $\Delta\mu H^+$ in regenerating rat-liver mitochondria which should therefore be evident in all uncoupled and energetically deflated mitochondria, is not clear. The former situation is most tenable since it is curious that the changes induced in F_1 associated with the mitochondrial inner membrane persist into the isolated complex, suggesting furthermore that one is not dealing predominantly with changes in F_1-F_0 interactions but also with changes within the F_1 moiety. A rather curious fact, however, is that ATP hydrolysis is not measurably diminished in F_1 isolated from regenerating rat liver compared to controls. Does this suggest that an interaction between F_1 subunits and the membrane F_0 sector is a necessary prerequisite to utilise the changes in nucleotide binding at the F_1 site? An interesting observation in [16], where changes in ATP-induced fluorescence in aurovertin bound to either ESMP or isolated F_0F_1 complex were not seen in F_1 alone, provides a very tempting analogy. The results and conclusions tendered in this report offer evidence of a physiological response to an abnormal stress situation, a response originating at the level of a discrete and subtle change in the interaction of a substrate with a site on the enzyme which is very likely to be non-catalytic and to exist for this very purpose.

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