Deficiency of Uncoupler-Stimulated Adenosine Triphosphatase Activity in Tightly Coupled Hepatoma Mitochondria

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ABSTRACT   Tightly coupled mitochondria from the well-differentiated hepatoma 7800 failed to exhibit a significant 2,4-dinitrophenol-activated ATPase activity at concentrations of uncoupler sufficient to completely inhibit oxidative phosphorylation. ATPase activity could not be maximally activated by uncoupling agents more potent than 2,4-dinitrophenol, such as carbonylcyanide p-trifluoromethoxyphenylhydrazone and 5-chloro, 3-tert-butyl, 2′-chloro, 4′-nitrosalicylanilide, nor by Mg^{++} after the following treatments: sonication, freezing, detergent lysis, and digestion with trypsin. Gel electrophoresis patterns of the membrane proteins of the hepatoma mitochondria revealed neither an absence of any one of the three different types of ATPase subunits characteristic of the homogeneous enzyme purified from normal liver mitochondria, nor a deficiency of the oligomeric molecule. Taken together, these data strongly suggest that the supramolecular structure of the membrane ATPase complex of mitochondria from hepatoma 7800 is altered in such a way that its capacity for ATP hydrolysis is severely diminished.

Well- and highly-differentiated hepatomas are currently being used in a number of different laboratories in an attempt to elucidate those molecular changes that accompany the conversion of a normal cell to a cancer cell. In contrast to many experimental tumors, well- and highly-differentiated hepatomas frequently grow slowly (1), have a karyotype very similar to that of the normal liver hepatocyte (2, 3), and show a normal rate of glycolysis (4, 5). Thus, biochemical differences between these hepatomas and normal liver are of great interest in that they may reflect a very early change in the transformation of a normal to a cancer cell.

In a recent communication from these laboratories (6), we reported that tightly coupled mitochondria can be prepared from the well-differentiated Morris hepatoma 7800. Mitochondria from this tumor exhibit high acceptor control ratios, 2,4-dinitrophenol-activated respiration, and appear to be morphologically and ultrastructurally intact when examined under the electron microscope. In this communication, we wish to report that the ATPase (ATP phosphohydrolyase, EC 3.6.1.3) in the membrane of tightly coupled mitochondria from hepatoma 7800 appears to be subject to severe inhibition. As will be described in detail, ATPase activity of freshly isolated mitochondria from this tumor cannot be maximally activated by highly potent uncouplers of oxidative phosphorylation or by Mg^{++} when these agents are added after disruptive treatments.

Abbreviations: DNP, dinitrophenol; MW, molecular weight.

MATERIALS AND METHODS

Chemicals. Pyruvate kinase (rabbit muscle), lactate dehydrogenase (beef heart), catalase (bovine liver), glyceraldehyde phosphate dehydrogenase (rabbit muscle), and cytochrome c (horse heart) were obtained from Sigma Chemical Co. Chymotrypsinogen (bovine pancreas) and trypsin inhibitor (soybean) were products of Worthington Chemical Co. Myoglobin (sperm whale) and trypsin (bovine pancreas) were purchased from Calbiochem. Hemoglobin was prepared from freshly drawn blood as described by Ingram (7). Lubrol WX was a product of I. C. I. America Inc.

Hepatomas. Induction and transplantation of hepatoma 7800 in male rats of the Buffalo strain were as described (8).

Acceptor Control and P/O Ratios. Acceptor-control ratios were determined as described by Chance and Williams (9). Respiration measurements were at 25°C in a closed 3.0-ml reaction vessel equipped with a Clark oxygen electrode. The respiration medium contained, in a final volume of 3.0 ml, 220 mM D-mannitol, 70 mM sucrose, 2.5 mM potassium phosphate, 2.0 mM N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid, 0.5 mM EDTA, 1.7 mM $MgCl_2$, 2.0 mg bovine plasma albumin, 10 mM sodium D-β-hydroxybutyrate, 2.5 mg mitochondria, and, when added, 152 μM ADP. The final pH was 7.4.

P/O ratios were determined by measurement of oxygen uptake and the formation of ^{32}P-labeled glucose-6-phosphate at 25°C in a system containing, in a total volume of 3.0 ml, 220 mM D-mannitol, 70 mM sucrose, 9.2 mM potassium phosphate, 2.0 mM N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid, 0.5 mM EDTA, 0.5 mM $MgCl_2$, 10.0 mM D-β-hydroxybutyrate, 0.75 mM ADP, 7.8 mM glucose, 44 international units of hexokinase, 0.10 μCi of ^{32}P-labeled phosphate, and 2.5 mg mitochondria. The final pH was 7.4. Extraction and counting of [^{32}P]glucose-6-phosphate were performed exactly as described (10).

ATPase Activity. Incubation before assay was for 4 min at 30°C in a medium containing, in a total volume of 0.450 ml, 11 mM imidazole, 55.5 mM sucrose and 0.5 mg mitochondria. ATP (disodium salt), 6.0 μmol, was then added, in a total volume of 0.150 ml. Further incubation was for 10 min at 30°C before the reaction was stopped with 0.10 ml of ice-cold 2.5 M $HClO_4$. After the quenched reaction mixture stood for 15 min at 0°C, 0.10 ml of 2.5 M KOH was added. Aliquots of 0.2 ml were removed and analyzed for orthophosphate by the colorimetric method of Gomori (11).
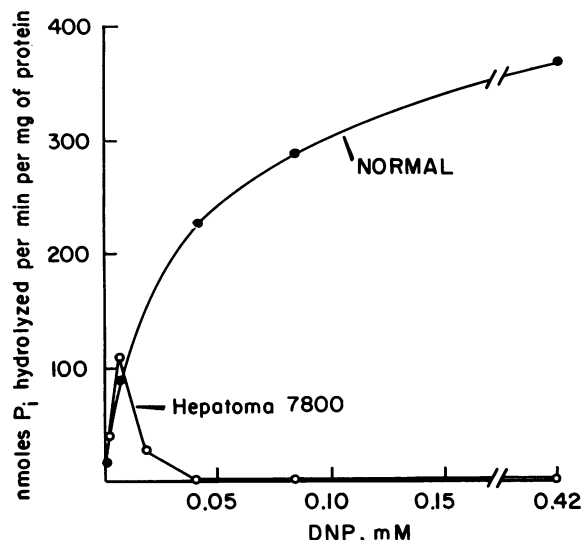
FIG. 1. Effect of DNP on the ATPase activity of normal-liver and hepatoma 7800 mitochondria.

Preparation of Mitochondrial Membranes. Mitochondria (25–50 mg/ml) in isolation medium were treated with a volume of Lubrol WX (19 mg/ml) such that the final suspension contained 0.16 mg of Lubrol WX/mg of mitochondrial protein. After standing at 0°C for 15 min, the suspension was diluted with isolation medium so that the final volume was twice the original volume of the mitochondrial fraction, then it was centrifuged at 35,000 rpm for 1 hr in the Spinco model L No. 40 rotor. The supernatant was carefully removed with a Pasteur pipette. The resultant sediment is essentially free of matrix and intracristal space activities and contains the inner and outer mitochondrial membranes (12).

Gel Electrophoresis. Electrophoresis of membrane proteins was performed exactly as described by Weber and Osborn (13), except that incubation prior to dialysis was in a medium containing 0.04 M sodium phosphate, 4% sodium dodecyl sulfate and 4% mercaptoethanol, pH 7.0. The following proteins were used as standards: bovine serum albumin, catalase, pyruvate kinase, lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, chymotrypsinogen, myoglobin and hemoglobin.

TABLE 1. *Comparison of the effect of DNP on oxidative phosphorylation and ATPase activity of mitochondria from normal liver and hepatoma 7800*

Mitochondrial Property	Normal		Hepatoma	
	Without DNP	With DNP*	Without DNP	With DNP*
Acceptor control ratio†	7.9	1.0	6.7	1.0
ADP/O ratio	2.7	0.0	2.6	0.0
P/O ratio	2.6	0.08	2.9	0.05
ATPase activity‡	82	386	64.5	49.5

* 8.3×10^{-5} M, final concentration in the assay.

† Rates of State 3 respiration were 31.5 and 27.8 natom oxygen per min per mg of protein for normal liver and hepatoma mitochondria, respectively.

‡ nmol P_i formed per min per mg.

Purification of ATPase. A homogeneous preparation of the ATPase of rat liver mitochondria was obtained after a sequence of purification steps consisting of sonication, washing in hypotonic buffer, and chromatography on DEAE-cellulose and Sephadex G-200. The purification scheme and properties of the purified enzyme will be published elsewhere.

Protein Determination. Protein was estimated by the biuret procedure.

RESULTS

Sensitivity of oxidative phosphorylation and ATPase activity to 2,4-dinitrophenol (DNP)

Data presented in Table 1 show that, in the presence of β-hydroxybutyrate as substrate, mitochondria isolated from hepatoma 7800 have acceptor control and P/O ratios very similar to those of normal (host) liver mitochondria. P/O ratios approach the theoretical value of 3.0 in both normal liver and tumor mitochondria, and are reduced to values near zero when 8.5×10^{-5} M dinitrophenol (DNP) is included in the assay. The ATPase activity of normal liver mitochondria is markedly activated in the usual manner (14) by 8.5×10^{-5} M DNP but, in sharp contrast to this activation, the ATPase activity of hepatoma mitochondria is actually inhibited by DNP (Table 1). Failure to observe a

TABLE 2. *Effect of uncoupling agents and disruptive treatments on ATPase activity of mitochondria from hepatoma 7800*

Addition or treatment	Concentration	ATPase activity*	Percent of maximal normal activity†
None	...	67	14.7
Mg^{++}	4.0 mM	52	11.9
DNP	8.3×10^{-5} M	50	11.0
DNP + Mg^{++}	4.0 mM, 8.3×10^{-5} M	61	13.4
Carbonylcyanide *p*-trifluoromethoxy-phenylhydrazone	1.6×10^{-6} M	50	11.0
5-Chloro,3-*tert*-butyl, 2'-chloro,4'-nitro-salicylanilide	1.6×10^{-6} M	50	11.0
Oleate	6.2×10^{-5} M	44	9.7
Lubrol‡¶	...	170	37.8
Frozen and thawed‡	...	171	38.0
Frozen and thawed + trypsin‡	...	182	40.5
Sonication‡	...	206	45.0

In disruptive treatments, mitochondria (25 mg/ml) were either frozen overnight at −20°C, or subjected to sonic oscillation at 15-sec intervals for 2 min with a small Biosonik probe set at 20% maximal intensity, 0–4°C. Digestion with trypsin was for 30 min at 30°C in a medium containing, in a final volume of 0.5 ml, 10 mM Tris-sulfate (pH 8.0), 1.0 mM EDTA, 950 μg of Lubrol WX, 5.0 mg of mitochondrial protein, and 15 μg of trypsin. The reaction was stopped by adding 75 μg of trypsin inhibitor in a volume of 0.05 ml.

* nmol P_i formed per min per mg.

† maximal activity of normal mitochondria was 450 nmol P_i per min per mg in the presence of either 4.0 mM Mg^{++}, after sonication as above, or in the presence of 8.3×10^{-5} M DNP.

‡ 4.0 mM Mg^{++} present.

¶ 190 μg of Lubrol WX was included in the assay.

significant activation of ATPase activity in hepatoma 7800 mitochondria by 8.5×10^{-5} M DNP was a consistent finding in eight different coupled preparations.

Effect of DNP concentration

Shown in Fig. 1 is the effect of DNP concentration on the ATPase activity of normal-liver and of hepatoma mitochondria, both of which exhibit similar acceptor-control properties. At all concentrations of DNP tested, the ATPase activity of normal mitochondria is significantly activated, whereas the ATPase activity of hepatoma mitochondria exhibits a biphasic dependence on DNP concentration. At very low concentrations of uncoupler (8.5×10^{-6} M), ATPase activity of the hepatoma mitochondria is partially activated (about 25% of maximal host activity), but at higher concentrations ATPase activity is completely inhibited. Thus, ATPase activity of hepatoma mitochondria is minimal in the presence of uncoupler concentrations that nearly maximally activate the latent activity in mitochondria from normal liver.

Effect of other uncouplers and disruptive treatments

Results summarized in Table 2 show that, in analogy to DNP (8.5×10^{-5} M), a number of other highly potent uncouplers of oxidative phosphorylation, including carbonyl-cyanide p-trifluoromethoxyphenylhydrazone, 5-chloro, 3-tert-butyl-2'-chloro, 4'-nitrosalicylanilide, and sodium oleate, fail to unmask significant ATPase activity in the hepatoma mitochondria. Combinations of DNP and Mg^{++} were also unable to unmask any ATPase activity in intact mitochondria from the hepatoma. Significant ATPase activity in hepatoma mitochondria could be activated by disruptive treatments such as freezing, detergent lysis with Lubrol WX, and sonication. In no case, however, did the ATPase activity unmasked by these disruptive procedures amount to more than 45% of the activity of normal liver mitochondria obtained in the presence of 8.5×10^{-5} M DNP (450 nmol P_i formed per min per mg). Enzymatic treatment of frozen and thawed hepatoma mitochondria with trypsin failed to elicit additional activity. Thus, not only is there a marked deficiency of uncoupler-activated ATPase activity in mitochondria freshly isolated from hepatoma 7800, but a marked deficiency of Mg^{++}-activated ATPase activity as well.

Comparison of the number and size distribution of membrane proteins of host and hepatoma mitochondria

Gel electrophoresis patterns of mitochondrial membranes from normal liver and hepatoma 7800 are summarized in Fig. 2, together with a gel electrophoresis pattern of the ATPase purified from normal rat liver mitochondria (manuscript in preparation). In general, the electrophoretic patterns of mitochondrial membranes of normal liver are very similar to those reported by Schnaitman (15), both with respect to the total number of proteins (about 23) and to the R_f values of individual proteins. With the exception of several minor differences noted in Fig. 2, electrophoretic patterns of tumor mitochondrial membranes are essentially identical to patterns obtained with normal liver membranes. Of most significance with respect to the thesis of this report is the finding that the protein bands that correspond to the three ATPase subunits of the homogeneous enzyme ($MW_A = 65,000$, $MW_B = 60,000$, $MW_C = 37,500$) (Table 3) are present in both normal and tumor preparations (Fig. 2 and Table 3) a finding that indicates that the deficiency of the ATPase
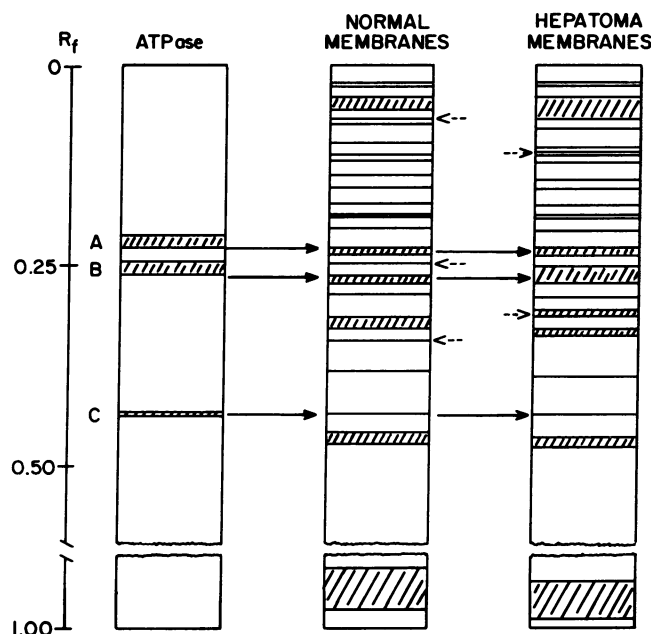

Fig. 2. Gel-electrophoresis patterns of the purified rat-liver ATPase, 20 μg, and membrane preparations of normal liver and hepatoma 7800 mitochondria, 200 μg, in the sodium dodecyl sulfate system (13). Bands corresponding to ATPase subunits are indicated by *dark arrows*. *Dotted arrows* indicate differences between mitochondrial membranes of normal liver and hepatoma and refer to proteins present in one membrane preparation but either absent or unresolved in the other. The mobility of a mixture of mitochondrial phospholipids was found to correspond very closely to the mobility of the broad band seen at the front of the gels that contained mitochondrial membranes. The patterns shown are averages of six samples run simultaneously.

activity in the tumor is not due to an absence of any one of the three ATPase subunits.

Close inspection of the gels, both visually (Fig. 2) and by scanning at 550 nm in the Gilford Spectrophotometer equipped with the Gilford automatic scanning attachment, revealed two other important pieces of information. First, it was found that the protein band corresponding to the B subunit of the ATPase is significantly broader in hepatoma than in normal patterns, a finding that might indicate a coalescence of the B subunit band with one or more of the missing bands noted in Fig. 2. Secondly, the sum of the areas under the peaks corresponding to the two principal ATPase subunits, A and B (not shown) was found to be slightly greater in the hepatoma than in the normal preparation, a finding that strongly indicates that the deficiency of ATPase activity

Table 3. *Molecular weights of the subunits of the purified ATPase of rat liver mitochondria, and of the proteins in normal-liver and hepatoma membranes that have mobilities similar to these subunits (see Fig. 2)*

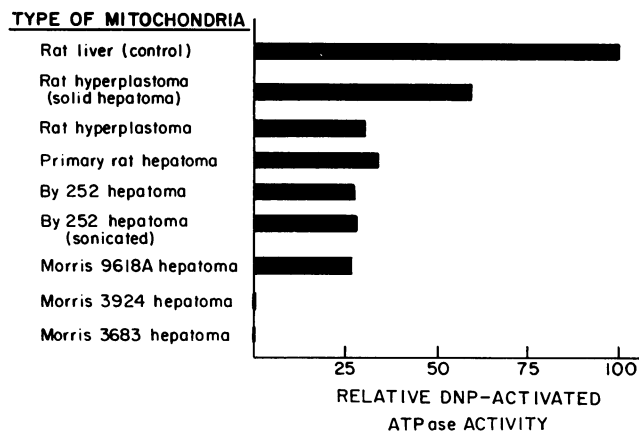| Band | Molecular weight | | |
	Purified ATPase	Normal membranes	Hepatoma membranes
A	65,000	64,500	64,000
B	60,000	58,300	59,000
C	37,500	38,500	39,000

TYPE OF MITOCHONDRIA

FIG. 3. Comparison of DNP-activated ATPase activity of mitochondria from several different hepatomas. Values for mitochondria from hepatomas not of the Morris variety were calculated from Tables 1, 2, 4, and 5 of Emmelot *et al.* (21). Where indicated, 10^{-4} M DNP was included in the assay. Data for mitochondria from Morris hepatoma 9618A was obtained in this laboratory in the presence of 8.5×10^{-6} M DNP. In the presence of higher concentrations of DNP, no ATPase activity was observed in mitochondria from hepatoma 9618A. Assay conditions were identical to those described for mitochondria from hepatoma 7800. Failure to observe DNP-activated ATPase activity in mitochondria from hepatomas 3924 and 3683 was reported by Devlin and Pruss (22).

in the tumor mitochondria is not due to a deficiency of the ATPase molecule. Insufficient resolving power of the available gel scanner did not permit a quantitative comparison of the areas under the A and B subunit peaks.

DISCUSSION

The unknown mechanism by which the energy of respiration is utilized to dehydrate ADP and P_i to give ATP is usually assumed to involve a number of reversible enzymatic steps (9, 16–18). The following lines of evidence, summarized in this report, strongly suggest that the reversibility of the energy-transfer system in mitochondria from hepatoma 7800 as measured by ATPase activity is markedly reduced, and that this reduction is not simply the result of a low molecular content of membrane-bound ATPase: (*a*) ATPase activity of intact tumor mitochondria is not markedly stimulated by uncouplers (Fig. 1, Table 2); (*b*) ATPase activity is not maximally increased by treatments or agents that normally disrupt the inner mitochondrial membrane (Table 2); (*c*) Gel electrophoresis patterns reveal similar amounts of the ATPase components in membranes of normal liver and hepatoma mitochondria (Fig. 2).

Although the failure to observe normal uncoupler-activated ATPase activity in hepatoma mitochondria may be subject to various explanations, two possibilities seem worthy of consideration. First, the low activity of uncoupler-activated ATPase could be attributed to a defective carrier or exchange diffusion system responsible for transporting ATP into mitochondria. Secondly, the ATPase molecule within the hepatoma membrane may be defective or inhibited. Of these two possibilities, aberrant transport seems least likely since the Mg^{++}-activated ATPase activity of hepatoma mitochondria, observed under conditions that normally disrupt the inner membrane, is also markedly reduced relative to normal-liver mitochondria. With respect to the second possibility, it is interesting to note that recent findings of Asami, Juntti, and Ernster (19), with beef heart mitochondria, suggest that the mitochondrial ATPase inhibitor of Pullman and Monroy (20) may be important in regulating the reversibility of the steps involved in ATP formation.

It would be of considerable interest to know whether the deficiency of ATPase activity observed in mitochondria from hepatoma 7800 is also a characteristic feature of other hepatomas. Although we have not examined this question in detail, we have found that tightly coupled mitochondria from Morris hepatoma 9618A, one of the most highly differentiated transplantable tumors available, are also highly deficient in uncoupler-activated ATPase activity (Fig. 3). Similarly, intact mitochondria isolated from several rapidly growing hepatomas by Emmelot *et al.* (21) and Devlin and Pruss (22) also fail to elicit a normal uncoupler-activated ATPase activity. The DNP-stimulated ATPase activity of mitochondria from these various hepatomas is compared in Fig. 3; in all cases it is seen to be at least 40% lower than the activity elicited by control-liver mitochondria.

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