Membranous Effects on Adenosine Triphosphatase Activities of Mitochondria from Rat Liver and Morris Hepatoma 3924A

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SUMMARY

Adenosine triphosphatase (ATPase) activities of sonically prepared submitochondrial particles of rat liver and Morris Hepatoma 3924A were compared as a function of changes in temperature. On Arrhenius plots, a discontinuity at 18°C was observed for the rat liver mitochondrial ATPase, while the hepatoma mitochondrial ATPase revealed a discontinuity at 20.4°C. Values for energy of activation of the rat liver and hepatoma mitochondrial ATPases were comparable below the break (34.5 and 35.5 kcal/mole, respectively) and above the break (11.6 and 9.2 kcal/mole, respectively). Solubilization of the mitochondrial membranes with Triton X-100 resulted in constant and similar values of energy of activation for the ATPases. Km values of hepatoma and rat liver mitochondrial ATPases for adenosine triphosphate were similar in both the membrane-bound and solubilized states. The lack of uncoupler-stimulated ATPase activity in hepatoma mitochondria is apparently not due to membranous effects on the affinity of the ATPase for adenosine triphosphate.

INTRODUCTION

The Morris hepatomas have served as useful sources for the biochemical characterization of effects of the tumorous state on cellular metabolic activities. One rather widespread feature of hepatoma mitochondria is that they fail to show stimulation of ATPase activity with uncouplers, such as 2,4-dinitrophenol and 5-chloro-3-tert-butyl-2′-chloro-4-nitosalicylanilide. The lack of uncoupler-stimulated ATPase activity may be due to a defective or inhibited ATPase in the mitochondrial membranes of the hepatomas (28), or there may be an alteration in the dinitrophenol-binding sites on the ATPase of tumor mitochondria (29, 35). Recently, Kaschnitz et al. (17) demonstrated uncoupler stimulation of hepatoma mitochondrial ATPase activities. However, stimulation of the hepatoma mitochondrial ATPase activities by dinitrophenol and 5-chloro-3-tert-butyl-2′-chloro-4-nitosalicylanilide was reduced if ATP was added to the assay medium after the uncoupler.

Alterations of mitochondrial membrane composition may greatly influence the expression of ATPase activity. Kagawa and Racker (16) demonstrated a phospholipid dependence on the expression of ATPase activity of the isolated oligomycin-sensitive ATPase, and reactivation of membrane-bound ATPase activity has been shown (5) to be regulated by chain length and degree of unsaturation of phospholipid acyl chains. Hepatomas provide natural sources for evaluating the effects of membrane compositional alterations (25, 32) on activities of membrane-bound enzymes. Van Hooven et al. (37) reported that, in rat hepatoma plasma membrane, the cholesterol content increased, and the degree of unsaturation of most lipid classes decreased. Bergelson et al. (1) reported that all membrane fractions of hepatoma cells underwent similar chemical dedifferentiation of their lipid components. Foe et al. (10, 12) noted that the ratio of cholesterol to phospholipid is higher in both the inner and outer mitochondrial membranes from various hepatomas (including Morris Hepatoma 3924A) than in mitochondrial membranes of adult rat livers. The change was due largely to increased cholesterol contents of the mitochondrial membranes.

Thus, because of the different properties of liver and hepatoma mitochondrial ATPases (e.g., response to 2,4-dinitrophenol), as a consequence of phospholipid dependency for ATPase activity, and since considerable differences in lipid composition in liver and hepatoma mitochondrial membranes have been described, we considered it important to examine for possible physical state differences of membrane lipids on the expression of ATPase activity in liver and hepatoma mitochondria. Experiments are also described that show that differences between rat liver and hepatoma mitochondrial membranes do not influence the Michaelis-Menten kinetic properties of the ATPases.
MATERIALS AND METHODS

Transplantation of Hepatoma. Seven-month-old female ACI/C were purchased from Laboratory Supply, Indianapolis, Ind. Morris Hepatoma 3924A was transplanted as previously described by Morris and Wagner (24). Tumors were harvested between 3 and 4 weeks after inoculation. Control rats did not receive inoculations of hepatoma cells.

Preparation of Mitochondria and Submitochondrial Particles. All preparatory procedures were conducted at 4°C. When the tumors reached the harvestable age, 3 tumor-bearing rats were decapitated by guillotine and bled. The hepatomas were removed from the rear legs; cleaned of fat, connective, and necrotic tissue; and then homogenized in 70 mm sucrose-220 mm mannitol-BSA3 (0.5 mg/ml)-HEPES buffer (0.5 mg/ml), pH 7.5, as described by Schreiber et al. (33). Livers from control rats were homogenized in a similar fashion. Mitochondria were isolated by differential centrifugation as described by Schreiber et al. (33).

Submitochondrial particles were prepared from freshly isolated mitochondria by resuspending the final mitochondrial pellets in 10 ml of 30 mm phosphate buffer, pH 7.05, and sonicating the suspensions for 1.5 min at 4°C at 20 kc with the use of a Biosonik III (Will Scientific, Rochester, N. Y.). The sonically prepared suspensions were centrifuged at 23,000 x g for 15 min to remove unbroken mitochondria. Submitochondrial particles were collected by centrifugation at 100,000 x g for 45 min and then resuspended in 250 mm sucrose-BSA (0.5 mg/ml)-2.0 mm HEPES buffer, pH 7.6, to a protein concentration of approximately 15 mg/ml. Prior to ATPase assays, the submitochondrial suspensions were further diluted to approximately 3 mg protein per ml in the same medium also containing 2 mm MgSO4.

Membranes of submitochondrial particles were solubilized by addition of 10% Triton X-100 to make a final concentration of Triton X-100 in the sample equal to 0.9%. The solubilized enzyme samples were held at room temperature.

ATPase Assays. ATPase activity was evaluated by following the oxidation of NADH at 340 nm in a coupled assay system with pyruvate kinase and lactate dehydrogenase (6), plus carbonyl cyanide m-chlorophenylhydrazone (1.0 /µM) in a Cary Model 14 spectrophotometer equipped with either a Haake or Forma Temp circulating, constant-temperature water bath. The bath water was circulated through the chamber wall of the sample compartment, and the temperature of the assay medium in the cuvet was determined with a small-surface thermistor probe attached to a YSI Model 42SC Tele-thermometer. Upon attainment of thermostoequilibrium in the cuvet, the reaction was started by addition of the protein sample.

Analytical Measurements. Protein concentrations were estimated by the method of Lowry et al. (23) with the use of BSA as a standard. ATP concentrations were determined from absorbance values at 260 nm, with a molar extinction coefficient of 15,400 (38).

Chemicals. ATP, NADH, BSA, pyruvate kinase, lactate dehydrogenase, oligomycin, atractylsine, and carbonyl cyanide m-chlorophenylhydrazone were purchased from Sigma Chemical Co., St. Louis, Mo. All other chemicals were of the best commercial grade.

RESULTS

Characterization of Mitochondrial ATPase Sources and Assay Conditions. Before examination of the kinetic characteristics of rat liver and hepatoma mitochondrial ATPases, it is necessary to comment first on the samples used in this study. The mitochondrial preparations were considered to be of comparable purity by examination of cytochrome c levels (13) and by examination of electron micrographs (G. Ojakian, personal communication).

Measurements of the membrane-bound ATPase activities were performed on sonically prepared submitochondrial particles in order to eliminate adenine nucleotide translocation as a limiting factor in ATPase activity estimations. The ATPase activities in our submitochondrial particle preparations were insensitive to atractylsine (10 µg/ml), a competitive inhibitor of adenine nucleotide translocation in mitochondria.

Solubilization of ATPase by addition of Triton X-100 was considered totally effective since such treatments rendered the ATPase activities insensitive to oligomycin (10 µg/ml), an inhibitor of membrane-bound ATPase.

The uncoupler, carbonyl cyanide m-chlorophenylhydrazone, was added to all ATPase assays of submitochondrial particles so that differences in rates of ATP hydrolysis between the liver and hepatoma samples would not be due to variations in the degree of coupling.

HEPES buffer was used in all of the ATPase assays because of its relatively lower temperature coefficient of ionization compared to other buffers that have significant buffering capacity at pH 8. Further to prevent temperature-induced pH changes in the buffer, we adjusted the pH of the reaction media buffer at various test condition temperatures.

ATPase activities in submitochondrial particles decreased significantly when held at 4°C in the sucrose-BSA-HEPES buffer. Within 3 hr the liver mitochondrial ATPase activity had decreased 18%, while a 27% decrease was noted for the hepatoma mitochondrial ATPase activity over the same time interval (Table 1). It was found that addition of Mg2+ to the submitochondrial particle suspensions promoted full retention of both the liver and hepatoma mitochondrial ATPase activities for over 5 hr. The solubilized enzymes were also fully active after 6 hr when held at room temperature in a medium containing Mg2+. No inhibition by oligomycin was observed.

Effect of Temperature on ATPase Activities. The effect of temperature on mitochondrial ATPase activities of rat liver and Morris hepatoma mitochondria is shown in Chart 1, as Arrhenius plots of the logarithm of ATPase-specific activities versus reciprocal of absolute temperature. This chart is a composite plot of data that was obtained from several experiments. Each point represents an average value obtained from at least 2 separate determinations. Straight lines of best fit were drawn after performing a least-squares linear regression of the data. Irreversible loss

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3 The abbreviations used are: BSA, bovine serum albumin; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
Table 1
Stability of rat liver and Morris Hepatoma 3924A mitochondrial ATPases

<table>
<thead>
<tr>
<th>Resuspending media</th>
<th>Rat liver* (%)</th>
<th>Hepatoma* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3 hr 5 hr</td>
<td>3 hr 5 hr</td>
</tr>
<tr>
<td>Control + 1.0 mm MgSO₄</td>
<td>-18 -22</td>
<td>-27 -27</td>
</tr>
<tr>
<td>Control medium was 250 mm sucrose-BSA (0.5 mg/ml)-2.0 mm HEPES buffer, pH 7.6.</td>
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Values represent the final changes in rates of ATP hydrolysis as compared to rates at 0 time.

Arrhenius plots of the membrane-bound mitochondrial ATPase activities of rat liver and Morris Hepatoma 3924A were evaluated from Lineweaver-Burk plots. The ATP hydrolysis reactions displayed typical Michaelis-Menten kinetics over the ATP concentration range of 0.3 to 3.0 mM. Kinetic constants of rat liver and Morris Hepatoma 3924A mitochondrial ATPase activities are tabulated in Table 2. The V_max values for the hepatoma mitochondrial ATPase were 3 times lower than were the V_max values for the rat liver mitochondrial ATPase. This difference was also seen in the specific activity values in the Arrhenius plots (Charts 1 and 2).

Table 2
Kinetic constants of rat liver and Morris Hepatoma 3924A mitochondrial ATPase activities

<table>
<thead>
<tr>
<th>Mitochondrial ATPase</th>
<th>K_m (ATP) (mM)</th>
<th>V_max (μmoles x min⁻¹ x mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Membrane-bound</td>
<td>0.51</td>
<td>3.0</td>
</tr>
<tr>
<td>Solubilized*</td>
<td>0.83</td>
<td>0.68</td>
</tr>
<tr>
<td>Morris Hepatoma 3924A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Membrane-bound</td>
<td>0.41</td>
<td>0.97</td>
</tr>
<tr>
<td>Solubilized*</td>
<td>0.70</td>
<td>0.19</td>
</tr>
</tbody>
</table>

Membranes were solubilized by addition of 10% Triton X-100 to make a final concentration of Triton X-100 in the sample equal to 0.9%.
The ATPase activities of rat liver and Morris Hepatoma 3924A were compared as a function of temperature. In both cases, discontinuities on Arrhenius plots were observed. The break temperature for rat liver mitochondrial ATPase was at 18°, while the break temperature for the hepatoma mitochondrial ATPase was at a slightly higher temperature (20.4°). The values for energy of activation of the membrane-bound enzymes were similar. Others (9, 26, 27, 31) have concluded that discontinuities on Arrhenius plots of membrane-bound enzymes are due to temperature-induced phospholipid phase changes from crystalline to liquid crystalline states. The implication that the break temperatures reflect specific lipid ATPase interactions is strongly supported by the observation that solubilization of the membrane with Triton X-100 resulted in linear slopes on Arrhenius plots for both rat liver and Morris Hepatoma 3924A mitochondria and yielded similar values for energy of activation.

The lipid environment in which membrane-bound enzymes are situated has been strongly implicated in enzymatic activity responses to changes in temperature. Cholesterol plays a dual role of increasing the viscosity of phospholipid hydrocarbon chains above transition temperatures, while below transition temperatures it prevents crystallization of hydrocarbon chains (7, 18, 19, 26, 31). Increasing the percentage of unsaturation of hydrocarbon chains tends to lower the temperatures at which phase transitions occur in membranes (2, 5, 14, 26). By interacting with membrane lipids, divalent cations (Mg²⁺ and Ca²⁺) tend to increase the temperature at which phase transitions occur (15, 36). In line with such considerations the cholesterol content of the inner mitochondrial membrane is about 5 times greater in Morris Hepatoma 3924A than in rat liver (12), and there is a significant decrease in the number of double bonds in hepatoma mitochondrial lipids (25). If such alterations in lipid composition occur within the microenvironment of the mitochondrial ATPase, then they may account for the discontinuities on the Arrhenius plots occurring at slightly different temperatures. In addition, the calcium content of the 3924A tumor is much greater than the content in normal rat liver (34). Thus, changes in lipid composition and/or changes in the cellular ionic environment may affect the fluidity of mitochondrial membrane lipids which in turn may exert regulatory control of ATPase activity.

There are numerous reports on the responses of mitochondrial membrane-bound enzymes to changes in temperature. Discontinuities on Arrhenius plots of mitochondrial ATPase activities have been observed; however, discrepancies can be found between various laboratories as to the temperature at which the discontinuity occurs. Temperature breaks of bovine heart mitochondria have been observed at about 17° (4, 22) and at 14.2° (5). Raison (30) reported a discontinuity for rat liver mitochondrial ATPase at about 23°. Lee and Gear (21) suggested that discontinuities on Arrhenius plots of rat liver mitochondrial ATPase activity occur with either an intact, energy-transducing membrane or when the activity is dependent on adenine nucleotide translocation. This suggestion was used to explain the lack of a discontinuity in the slope of Arrhenius plots of ATPase activity of sonicated rat liver mitochondria. In our studies, the ATPase activity in sonically prepared submitochondrial particles was examined in the presence of the uncoupler, carbonyl cyanide m-chlorophenylhydrazone. Although the mitochondria were sonically disrupted and the particles were totally uncoupled, we still observed discontinuities on Arrhenius plots. We attribute these discontinuities to phase changes of the lipids of the mitochondrial membranes. Feo et al. (11) reported a break temperature at 24° for rat liver mitochondrial ATPase and a break temperature at 23° for Yoshida Hepatoma AH-130 mitochondrial ATPase. The differences in their results and ours may be due to differences in preparation of the membrane samples or differences in the strain of rats and hepatomas.

In a number of studies there has been observed (3, 9, 19, 22, 26, 36) a lack of correspondence between lipid-phase changes detected by physical measurements (e.g., electron spin resonance, differential scanning calorimetry, X-ray diffraction) and transition temperatures detected by estimating enzyme activities as a function of temperature. Transitions of multiple enzymes on specific membranes also do not always occur at the same temperature. These observations have led to the suggestion that heterogeneous distributions of lipids exist within various biological membranes. Thus, the lipid composition in the microenvironment around one enzyme may be significantly different than the lipid microenvironment about another enzyme in the same membrane. Likewise, the microenvironments about the rat liver and the hepatoma mitochondrial ATPases may be different since the membrane-bound forms elicited slightly different break temperatures. Membrane lipid compositional differences are most probably responsible for altered microenvironments of the ATPase.

Membrane differences between rat liver and hepatoma mitochondria apparently do not affect the Km values of the ATPases for ATP. Our Km values were essentially the same for rat liver and hepatoma mitochondrial ATPases in both the membrane-bound and solubilized states. The lack of uncoupler-stimulated ATPase activity in hepatoma mitochondria thus cannot be accounted for by an effect of the membrane on the affinity of the ATPase for ATP.

The Vmax value of the rat liver mitochondrial ATPase was about 3 times greater than the Vmax value of the hepatoma mitochondrial ATPase. Generally, such differences can be accounted for by different amounts of active enzyme or because of altered interactions between the enzyme and...
the membrane. It is unlikely that the membrane exerts a differential latent effect on the hepatoma ATPase activity of submicrobial particles since solubilization of the enzyme by Triton X-100 did not promote an increase in activity.

REFERENCES


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