Enzymatic and Spectral Analysis of Cytochrome Oxidase in Adult and Fetal Rat Liver and Morris Hepatoma 3924A


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SUMMARY

Cytochrome oxidase, when measured either spectrally as cytochrome a and a3 or polarographically by following the enzymatic oxidation of reduced cytochrome c, was about 60% lower in whole-cell suspensions of Morris hepatoma 3924A than in whole-cell suspensions of normal or host rat liver. Fetal liver, which has a maturation time very near that of the 3924A hepatoma, had a cytochrome oxidase activity about 50% lower than control liver. The cytochrome oxidase content of mitochondria isolated from the tumor cells was significantly higher than that of control liver mitochondria by use of either the spectral or polarographic assay method. Thus, the marked deficiency of cytochrome oxidase in the 3924A tumor cells could be ascribed most easily to a low mitochondrial content in the cell rather than to a reduced or impaired terminal cytochrome system in mitochondria. The compatibility of the spectral and polarographic assay methods for assessing the relative amounts of cytochrome oxidase in normal and neoplastic tissue are clearly demonstrated by this study.

INTRODUCTION

Since Warburg's early studies (21), which showed that many cancer cells exhibit a high glycolytic capacity and a low rate of respiration, a number of investigators have designed experiments to measure either the mitochondrial or cytochrome content of cancer cells. In general, the mitochondrial content of tumor cells has been shown to be significantly lower than that of normal tissue (1, 3, 4, 14–18, 20, 22). Experiments carried out to establish the cytochrome content of tumors, however, have given a less consistent picture. Measurement of cytochrome oxidase by enzymatic methods suggests that this respiratory carrier is either deficient or impaired in many tumors (8), whereas spectral measurements reveal a rather normal cytochrome oxidase content in those tumors examined (5, 6). Unfortunately, in most investigations of the cytochrome oxidase content of tumors, both types of assay methods have not been used concurrently.

Experiments described in this communication were designed to provide information about the cytochrome oxidase content of Morris hepatoma 3924A relative to normal, host, and fetal liver by using both the oxygen electrode enzymatic assay (polarographic assay) and the spectral assay. As will be described below, both assay methods are compatible and clearly show that the cytochrome oxidase content is significantly reduced in Morris hepatoma 3924A relative to control liver. In addition, experiments described below indicate that the cytochrome oxidase activity of fetal liver cells is very close to that of the 3924A hepatoma.

MATERIALS AND METHODS

Rats. ACI/T control and 3924A tumor-bearing male rats were supplied by Dr. H. P. Morris. The induction and transplantation of this rat hepatoma have been described in detail elsewhere (11). Sprague-Dawley males and females (Bar F Rabbitry, Baltimore, Md.) were mated in our laboratory to supply the fetal material. The females were placed with males on the day of proestrus as determined by a vaginal smear. Mating was confirmed by the presence of sperm in the smear taken on the following day. The animals were maintained on food and water ad libitum and were housed no more than 5/cage.

Preparation of Whole-Cell Suspensions. Control, tumor-bearing, and pregnant rats were anesthetized by an i.p. injection of 0.4 ml of Nembutal (50 mg/ml, Abbott Laboratories, North Chicago, Ill.). Tumors were dissected from the leg musculature, freed of connective tissue and necrotic areas, and placed in ice-cold Locke's citrate solution (9.5 g NaCl, 75 mg KC1, 150 mg NaHCO3, 1 g glucose, and 0.027 M sodium citrate in 1 liter of conductivity water, pH 7.6). The livers of control and tumor-bearing animals were perfused with 50 ml of Locke's citrate at 37°. The blood-free livers were placed in ice-cold Chance's "saline phosphate" (6). Fetuses of 17 to 19 days of gestation were removed from the uterine horns, and the fetal livers were dissected free and placed in ice-cold Locke's citrate. The tumor and liver materials were put through an ice-cold tissue press (Harvard Apparatus, Inc., Dover, Mass.), and the resulting mince was filtered through 10 X 10 mesh (nylon) by adding 50 ml ice-cold Locke's citrate. The filtrate was centrifuged at 70 X g for 2 min. The pellets were washed twice with ice-cold Locke's citrate, each...
time centrifuging 2 min at 70 X g. Because of contamination by red blood cells, the fetal liver material was washed 4 or 5 times. The final pellets contained isolated whole cells free of blood cells as confirmed by light microscopy.

Preparation of Mitochondria. Control and tumor-bearing rats were decapitated with a guillotine and allowed to bleed 3 or 4 min. Livers and tumors were removed; cleaned of fat, connective tissue, and necrotic areas; and placed in ice-cold H-medium (23.9 g sucrose, 38.2 g mannitol, 0.5 g N,N'-hydroxyethylpiperezine N'-ethanesulfonic acid, and 0.5 g bovine serum albumin in 1 liter of conductivity water, pH 7.4). Since relatively few mitochondria could be isolated from 3924A tumors, the tumors from 3 animals were combined and treated as 1 sample. Similarly, the livers from the same 3 animals were combined and treated as 1 sample. Tumors and livers were cut into small pieces, washed twice with 30 ml H-medium, and then suspended in 2 volumes of medium. The mince was homogenized with 4 passes of a motor-driven Potter-Elvehjem tissue grinder and diluted with H-medium to give a 10% homogenate. The homogenate was centrifuged in a refrigerated Sorvall centrifuge (0—4°) at 660 x g for 15 min. The supernatant was carefully decanted and centrifuged again at 660 x g for 15 min. The resultant supernatant was then centrifuged at 6780 x g for 15 min to give a tightly packed mitochondrial pellet. Any fluffy material was carefully discarded. The mitochondrial pellet was resuspended in one-half the original centrifugation volume of H-medium and centrifuged at 9770 x g for 15 min. The pellet was resuspended a final time in one-fourth the original centrifugation volume and centrifuged again at 9770 x g for 15 min.

Enzymatic Assay of Cytochrome Oxidase. The assay mixture contained, in a final volume of 2.0 ml, 73 mM sodium phosphate (pH 7.4), 3.8 mM sodium ascorbate, 0.3 mM N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride, 0.05 mM cytochrome c [Sigma Type III, concentration determined by absorbance at 550 nm, assuming an extinction coefficient of \(27.6 \text{ mM}^{-1} \text{cm}^{-1}\) (12)] and 0.25 to 0.50 mg of a Lubrol-treated tissue or mitochondrial sample. Prior to assay, tissue or mitochondrial samples were treated with 1.0 ml of 1.9% Lubrol WX (I. C. I. America Inc., Stamford, Conn.) to disrupt the cell and/or mitochondrial membrane (13). Cytochrome c was added to initiate the reaction. The rate of oxygen uptake was followed polarographically at 25° with a Clark oxygen electrode.

In control experiments, the cytochrome oxidase assay was found to be linear both with respect to time and tissue or mitochondrial protein concentration. For the levels of tissue or mitochondria used (0.25 to 0.50 mg), 0.05 mM cytochrome c was sufficient to give a maximal velocity response. Samples of Lubrol-treated cells and mitochondria, when added to the same assay medium, gave an additive velocity response demonstrating that Lubrol-treated whole cells do not contain an inhibitor of cytochrome oxidase.

Spectral Assay of Cytochrome Oxidase. Concentration of cytochrome a and a3 in whole-cell or mitochondrial samples was determined from difference spectra obtained with a split-beam, wavelength-scanning spectrophotometer. Samples (1.0 ml) containing 10 to 12 mg of protein in the case of liver and tumor cell suspensions, and 4.0 mg in the case of liver and tumor mitochondrial suspensions, were placed in 2 quartz cuvets of 1.0-cm light path. A baseline was obtained by automatically scanning the spectrum between 630 and 400 m\(\mu\) and recording the absorbance ratio of the 2 cuvets. A difference spectrum for each sample was obtained by recording the difference in absorption between fully oxidized cytochromes (oxygen bubbled through 1 cuvet) and fully reduced cytochromes (dithionite added to the other cuvet). The concentration of cytochrome a was calculated from the absorbance difference between the measuring wavelengths 605 and 625 m\(\mu\) and the extinction coefficient for cytochrome a, 16 mM\(^{-1}\) (7). The concentration of cytochrome a3 was calculated from the absorbance difference between the measuring wavelengths 444 and 455 m\(\mu\) and the extinction coefficient for cytochrome a3 90 mM\(^{-1}\) (7).

Other Experimental Methods. The cell number in cells/ml of whole-cell preparations was estimated with a hemocytometer (Spencer, A. O. Instrument Co., Buffalo, N. Y.). The concentration of protein in normal, host, and tumor whole-cell suspensions was determined by the method of Lowry et al. (10).

Statistical Analysis. Probability (p) values were derived by Student's t test (19).

RESULTS

Whole-Cell Spectra. In order to determine whether cytochrome oxidase in whole cells could be calculated from spectral measurements, suspensions of liver and tumor cells were subjected to difference spectra analysis (Chart 1). The difference spectrum of the tumor shows the same peaks as those obtained by Estabrook and Holowinsky (7) on isolated liver mitochondria. Although the difference spectrum of whole liver cells differs somewhat in the a-region around 550 m\(\mu\), the a (+a3) peaks of cytochrome oxidase and the isosbestic points on either side of these peaks are essentially the same as those found in the isolated mitochondrial spectrum (cf Chart 2). No carboxyhemoglobin peaks appeared in difference spectra of the reduced versus the carbon monoxide-treated whole-cell samples. Therefore, the absorption peaks of the whole-cell difference spectra presented in Chart 1 reflect only cytochrome-absorbing components, and can be used to estimate the cellular content of cytochrome oxidase without interference by hemoglobin.

Cytochrome Oxidase Content of Whole Cells. Results of enzymatic and spectral cytochrome oxidase assays on intact cell suspensions are presented in Table 1. Calculations of cytochrome oxidase as cytochromes a and a3 from spectral measurements show a significant deficiency of this terminal enzyme in the 3924A cell relative to normal or host liver. Thus the respective contents of cytochrome a and a3 (moles/mg protein) are only 44 and 64% that of control tissue. Enzymatic determination of the relative amounts of cytochrome oxidase gave results similar to the spectral measurements; the specific activity of cytochrome oxidase in tumor cells (μatoms oxygen consumed/mg protein/min) is about 40% that of control tissue.
Fetal liver, which has a maturation time very near that of the 3924A hepatoma, was also found to exhibit a low cytochrome oxidase activity. The specific activity of cytochrome oxidase of fetal cells (230 ± 15 μatoms oxygen consumed/mg protein/min) lies between that of control liver (487 ± 52) and tumor (193 ± 54), although much closer to tumor. Due to lack of material, spectral data were not obtained on fetal cells.

Data presented in Table 2 show that on a per cell basis the cytochrome oxidase content of 3924A tumor cells is less than 5% that of host liver cells. When viewed microscopically, however, tumor cells were found to be noticeably smaller than host liver cells. Thus 1.0 mg of tumor contains (5.3 ± 1.1) × 10⁶ cells, whereas 1.0 mg of host liver contains (5.0 ± 0.8) × 10⁶ cells. These data as well as those presented in Table 1 clearly show that the 3924A Morris hepatoma, when compared with its tissue of origin, is markedly deficient in the terminal cytochrome of the mitochondrial electron transport chain.

**Cytochrome Oxidase Content of Mitochondria.** In order to determine whether the deficiency of cytochrome oxidase in...
the Morris hepatoma 3924A cell is due to a low cellular content of mitochondria or a low cytochrome oxidase content in the mitochondria, enzymatic and spectral assays were performed on isolated mitochondria from tumor and host liver. Difference spectra presented in Chart 2 clearly show that tumor and host mitochondria are qualitatively identical, in that they contain the same cytochrome-absorbing components.

Calculations of the cytochrome oxidase content from spectral measurements failed to reveal a deficiency of this terminal cytochrome in the isolated tumor mitochondria (Table 3). On the contrary, the cytochrome oxidase content (moles cytochrome $a$ and $a_3$/mg protein) was found to be significantly higher in mitochondria than in mitochondria isolated from either normal or host liver. Similarly, enzymatic assay of cytochrome oxidase showed a significantly higher activity in tumor mitochondria than in host liver mitochondria. These results strongly suggest, therefore, that the low cytochrome oxidase content of the 3924A tumor is a consequence of a low mitochondrial population within the cell itself, rather than a low cytochrome oxidase content within the mitochondria.

DISCUSSION

Chance and Hess (5, 6), using the spectral assay for determining cytochrome oxidase, showed that ascites tumor cells have a cytochrome oxidase content comparable to other actively respiring tissues that they had studied. These spectroscopic studies raised some doubt about the validity of enzymatic cytochrome oxidase assays on whole-cell suspensions (2), and, moreover, suggested that many rapidly growing tumors, previously shown to have a low cytochrome oxidase content with the enzymatic assay, might in fact have a fairly normal level of this terminal cytochrome. Results of experiments on the rapidly growing 3924A Morris hepatoma presented here would suggest that the enzymatic assay for cytochrome oxidase probably gives as reliable an indication of the relative level of cytochrome oxidase as does the spectral assay. Thus, the cytochrome oxidase content of the 3924A hepatoma relative to control liver was found to be greatly reduced with either method (cf. Tables 1 and 2).

Although the cytochrome oxidase content of the ascites cell has been considered to be at variance with the relative levels of this cytochrome found in other rapidly growing tumors, this

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**Table 2**

Cytochrome oxidase content of whole cells expressed on a per cell basis

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cell type</th>
<th>moles $a$ cell $\times 10^{17}$</th>
<th>moles $a_3$ cell $\times 10^{17}$</th>
<th>mmol O$_2$ consumed cell $\times$ min $\times 10^{5}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Host liver</td>
<td>22.8 ± 3.0</td>
<td>18.3 ± 2.9</td>
<td>97.6 ± 14.1</td>
</tr>
<tr>
<td>2</td>
<td>3924A tumor</td>
<td>0.95 ± 0.31$^b$</td>
<td>1.12 ± 0.22$^b$</td>
<td>3.65 ± 1.02$^b$</td>
</tr>
</tbody>
</table>

$^a$Values are means ± S.D.

$^b p < 0.001$ compared to host liver.

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**Table 3**

Cytochrome content of isolated mitochondria

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Mitochondrial type</th>
<th>No. of experiments</th>
<th>moles $a$ mg protein $\times 10^{10}$</th>
<th>moles $a_3$ mg protein $\times 10^{10}$</th>
<th>mmol O$_2$ consumed mg protein $\times$ min $\times 10^{3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal liver</td>
<td>3</td>
<td>2.26 ± 0.27</td>
<td>1.83 ± 0.33</td>
<td>1.15 ± 0.01</td>
</tr>
<tr>
<td>2</td>
<td>Host liver</td>
<td>6</td>
<td>2.20 ± 0.43</td>
<td>1.71 ± 0.28</td>
<td>1.30 ± 0.11</td>
</tr>
<tr>
<td>3</td>
<td>3924A tumor</td>
<td>6</td>
<td>3.50 ± 0.75$^b$</td>
<td>3.10 ± 0.94$^c$</td>
<td>1.80 ± 0.25$^b$</td>
</tr>
</tbody>
</table>

$^a$Values are mean ± S.D.

$^b p < 0.01$ compared to host liver.

$^c p < 0.02$ compared to host liver.
may not be the case. Since the ascites cell has no appropriate tissue of origin, Chance and Hess (5, 6) compared the cytochrome oxidase content of this tumor with values obtained on yeast and muscle. They found the cytochrome oxidase content of the ascites tumor (7 to 9 moles/g cell X 10^9) to lie between that of yeast (20 moles/g cell X 10^9) and muscle (2 moles/g cell X 10^9). However, the values for these latter 2 tissues are so widely different that the significance of such a comparison is difficult to evaluate. In most other studies of rapidly growing tumors, including the one summarized in this communication, the cytochrome oxidase content of the tumor has been more appropriately compared to that of its tissue of origin.

The 3924A hepatoma and fetal liver both have rapid growth and glycolytic rates; the cytochrome oxidase activity of both tissues is similarly low relative to control liver (cf. Tables 1 and 2). An extension of the studies reported here to other tumors of varying growth and glycolytic rate would be valuable in establishing to what extent the mitochondrial content of tumors is related to these parameters. At present, such studies are impeded by the fact that the more slowly growing tumors, unlike the 3924A tumor, contain a large number of red blood cells, the hemoglobin of which markedly interferes with the spectral assay. With the advent of improved techniques for preparing blood-free and hemoglobin-free tumor cell suspensions, these studies can hopefully be extended.

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REFERENCES

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