ELECTRON PARAMAGNETIC RESONANCE STUDIES OF IRON-SULFUR CENTERS IN MITOCHONDRIA PREPARED FROM THREE MORRIS HEPATOMAS WITH DIFFERENT GROWTH RATES*

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SUMMARY. Iron-sulfur centers in mitochondria prepared from Morris hepatomas with different growth rates were compared with those in host liver and non-tumor-bearing rat liver mitochondria by EPR measurements (< 77° K). In the slow growing hepatoma 16, EPR signals from iron-sulfur centers located in the NADH dehydrogenase region were specifically diminished. In the rapidly growing hepatoma 7777, EPR signals of all the iron-sulfur centers showed considerably diminished intensity. In hepatoma 7800 having an intermediate growth rate, all iron-sulfur centers showed no change. Those changes in iron-sulfur centers correlated with observed respiratory activities of Morris hepatoma mitochondria. No general correlation was obtained between these parameters and the growth rate of the tumors.

EPR signals from a large number of iron-sulfur centers have recently been detected in mammalian, avian and yeast mitochondria by Ohnishi et al. (1-3), and Orme-Johnson et al. (4,5), using electron paramagnetic resonance (EPR) techniques at temperatures between that of liquid nitrogen (77° K) and liquid helium (4.2° K). The low temperature (< 77° K) EPR technique represents a specific and sensitive tool for the study of individual iron-sulfur centers in the respiratory chain. As illustrated in Fig. 1, at least seven iron-sulfur centers have been previously reported in the NADH dehydrogenase region (2-10), two iron-sulfur centers in the succinate dehydrogenase region (12), and two in the cytochrome bc_1 region (14,15). It may be noted that probably each iron-sulfur center contains two or four atoms of iron and acid labile sulfur, respectively. The role of most of these iron-sulfur centers as electron carriers in the respiratory chain has been suggested from kinetic

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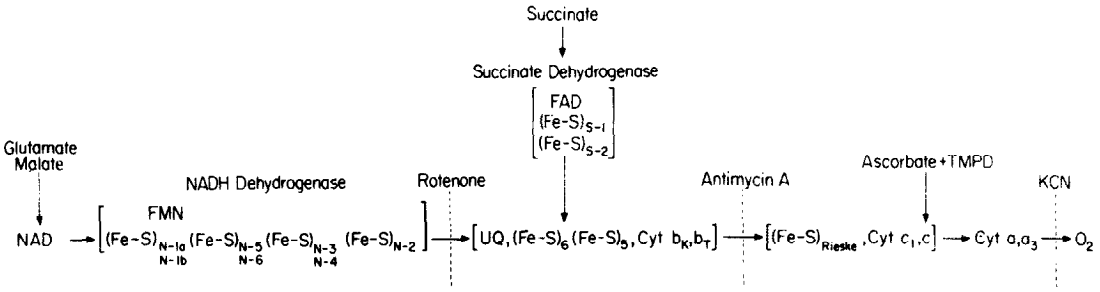

Figure 1. Tentative scheme of respiratory chain components and reaction sites of respiratory inhibitors. The $(Fe\text{-}S)_{N-1}$ .... $(Fe\text{-}S)_{N-6}$ are designated according to Orme-Johnson et al. (4,5) and Ohnishi et al. (6,7). Iron-sulfur centers associated with NADH dehydrogenase (8-10) are nominated here with the prefix N. $(Fe\text{-}S)_{S-1}$ and $(Fe\text{-}S)_{S-2}$ are iron-sulfur centers associated with the succinate dehydrogenase; the $(Fe\text{-}S)_{S-1}$ signals are detectable at 77° K (11), while the $(Fe\text{-}S)_{S-2}$ signals are detectable only below 20° K (12). $(Fe\text{-}S)_{S-2}$ is probably not directly involved in the respiratory chain. $(Fe\text{-}S)_6$ is an iron-sulfur center (g_1 = 2.11, g_2 = 1.90) with the half-reduction potential around OmV, but its function and location are unknown (13).

studies of their redox reactions using cardiac submitochondrial particles (16), purified NADH dehydrogenase (17), succinate dehydrogenase (18), and from an observed close correlation between the maximal rate of electron transfer and relative intensity of EPR signals arising from iron-sulfur centers associated with different dehydrogenase regions of the respiratory chain, using various yeast systems (13,19).

Pedersen et al. (20) and Schreiber et al. (21) reported some characteristics of mitochondria prepared from three Morris hepatomas of widely different growth rates. These investigators obtained the following results: (a) mitochondria from the rapidly growing hepatoma 3924A did not oxidize the NAD-linked substrate, β-hydroxybutyrate, while succinate was oxidized with a normal respiratory rate but with a diminished phosphorylation efficiency; (b) mitochondria from slow (9618A) and intermediate (7800) growth rate hepatomas respired normally in the presence of either β-hydroxybutyrate or succinate and showed normal phosphorylation efficiency; (c) both cytochrome oxidase and malate dehydrogenase (as representative of mitochondrial enzymes) activities were near normal in all hepatoma mitochondria examined.

LaNoue et al. (22), on the other hand, reported that respiratory control ratios were near normal in mitochondrial preparations from Morris hepatomas having a wide variety of growth rates (7777, 7800, 7794A and 16), but did observe diminished rates of both NAD-linked substrate oxidation and succinate-oxidation in mitochondria prepared from both slow and fast growing hepatomas. Observations reported by both groups of investigators suggested

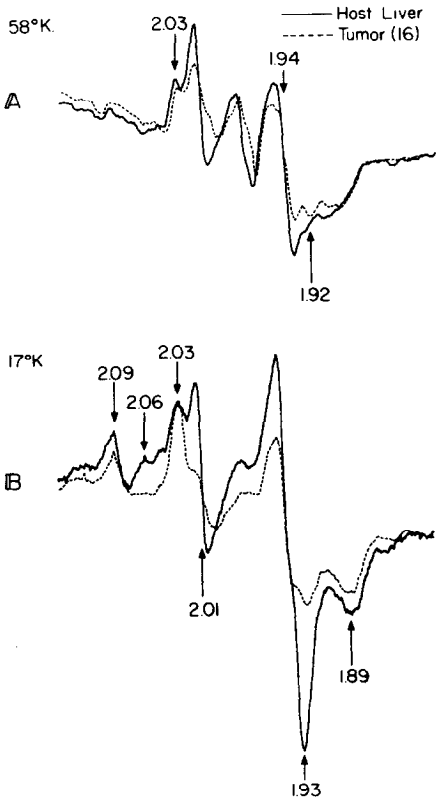

Figure 2. EPR spectra of reduced iron-sulfur centers in mitochondria prepared from Morris hepatoma 16 and host liver, measured at 58° K and 17° K. Mitochondrial suspensions were brought to anaerobiosis by incubating with 8.3 mM glutamate plus 8.3 mM malate for 10 minutes at room temperature after transferring into EPR tubes. Protein concentrations of both tumor and host liver mitochondria were 60 mg/ml. EPR operating conditions were: modulation frequency, 100k Hz; modulation amplitude, 12.5 gauss; microwave power, 50 mW; microwave frequency, 9.113 GHz; time constant, 0.3 sec; scanning time 500 gauss per minute. EPR spectra were recorded at temperatures indicated in the figure.

some defects of the respiratory components in the Site I and II regions of the respiratory chain in Morris hepatoma mitochondria. In the present investigation, the respiratory rate of host liver mitochondria and mitochondria from three kinds of Morris hepatomas (7777, 7800, 16) oxidizing glutamate plus malate, succinate, or TMPD plus ascorbate has been compared with the relative intensity of EPR signals from iron-sulfur centers in different segments of the respiratory chain.

MATERIALS AND METHODS

Non-tumor-bearing and tumor-bearing Buffalo strain rats were obtained from Dr. Harold P. Morris. Morris hepatomas 16 (slow growth rate), 7800 (intermediate growth rate) and 7777 (rapid growth rate) were used (23).

TABLE I

Respiration Rates in Mitochondria Prepared from Morris Hepatoma, Host Liver and Non-Tumor-Bearing Rat Liver

Mitochondria were incubated in a medium containing 150 mM KCl, 20 mM Tris-HCl, 10 mM phosphate-Tris, 0.1 mM EDTA, 5 mM $MgCl_2$ and 30 mM glucose (pH 7.2). Mitochondrial protein was 1 to 4 mg/ml of reaction media. Various metabolic states were obtained as follows: State 4, no additions to the basic mixture other than substrate; State 3, additions to the basic mixture include substrate, 100 µM ADP, 8 units of hexokinase (Type F-300, Sigma Chemical Company); Uncoupled, additions to the basic mixture include substrate and P-trifluoromethoxyphenylhydrazone of carboxyl cyanide (0.2-0.3 nmoles/mg protein). Substrate concentrations were 12.5 mM glutamate, 5 mM malate, 10 mM succinate plus 5 µM rotenone, and 2 mM ascorbate plus 0.9 mM tetramethylphenylene diamine (TMPD). Values are means ± standard error, and number of determinations are shown in parenthesis.

Preparation	Glutamate-Malate				Succinate-Rotenone				Ascorbate-TMPD	
		State 3	State 4	Uncoupled		State 3	State 4	Uncoupled		Uncoupled
		natoms O/min·mg								
Control liver	(4)	78.7±4.6	10.2±0.6	124.3±7.1	(4)	102.5±5.4	16.5±1.5	149.3±7.0	(4)	164.5±3.4
Host liver	(41)	88.4±2.6	12.9±0.8	123.4±4.1	(25)	117.4±3.6	24.5±1.4	164.7±4.1	(25)	166.2±3.1
Tumor 16	(12)	38.3±1.8	8.6±0.4	51.9±2.5	(10)	83.0±2.1	23.3±1.8	108.4±4.4	(8)	144.1±13.5
Tumor 7800	(13)	78.3±4.2	11.4±0.9	109.3±6.0	(7)	99.1±7.0	26.2±3.4	119.0±9.2	(5)	180.7±9.0
Tumor 7777	(12)	47.9±7.1	11.1±1.7	76.9±7.6	(7)	38.5±6.0	14.3±1.6	58.0±9.2	(5)	83.5±10.4

Mitochondria were prepared according to LaNoue et al. (24). Respiration was measured polarographically using a Clark oxygen electrode. EPR measurements were performed on a Varian Model E-4 spectrometer. The samples were contained in quartz sample tubes 3 mm in diameter and rapidly frozen by immersion in liquid isopentane at 113° K. The sample temperatures below 77° K were obtained by cooling the samples with a stream of cold helium gas derived from boiling liquid helium. The temperature was measured by an Au/Co versus Pt thermocouple (1).

RESULTS

Respiratory Rate with Various Substrates in Morris Hepatomas and Their Host Liver Mitochondria. Respiratory rates with various substrates in liver mitochondria of tumor-bearing rats showed no significant change from those in liver mitochondria of non-tumor-bearing rats (Table I). Thus, as a routine procedure, mitochondria from hepatomas and host livers were prepared simultaneously under the same conditions, and respiration rates were compared. All mitochondrial preparations had a normal phosphorylation efficiency, and had respiratory control ratios between 4 and 8. For the comparison of electron transfer rates in different spans of the respiratory chain, respiratory rates in the presence of glutamate plus malate, succinate, or ascorbate plus TMPD were measured in State 3, State 4, and in the uncoupled state. In mitochondria prepared from the slow growing hepatoma 16 (Table I), glutamate plus malate respiration showed the most diminished rate (57% decrease); succinate respiration showed a less diminished rate (29% decrease) while ascorbate plus TMPD respiration was not different from that in host liver mitochondria. In mitochondria prepared from Morris hepatoma 7800 of intermediate growth rate, there were no significant changes in respiratory rates from those in host liver mitochondria with all substrates examined. In the rapidly growing hepatoma 7777, mitochondrial respiration was decreased with all substrates tested.

Iron-sulfur Centers in Mitochondria Prepared from Morris Hepatomas and Host Liver. No significant difference of line shape and signal intensity was observed between EPR spectra of reduced iron-sulfur centers in liver mitochondria prepared from tumor-bearing and non-tumor-bearing rats. Thus, EPR signals of iron-sulfur centers reduced with glutamate plus malate in tumor mitochondria were compared with corresponding signals in host liver mitochondria. The results which are presented here are representative of three preparations of each host liver and hepatoma mitochondria studied.

Fig. 2 shows the EPR spectra of reduced iron-sulfur centers in mitochondria prepared from Morris hepatoma 16 denoted by the dotted line, and host liver denoted by the solid line. As seen in the spectrum from the host liver mitochondria, the so-called "g = 1.94" signals which arise from both iron-

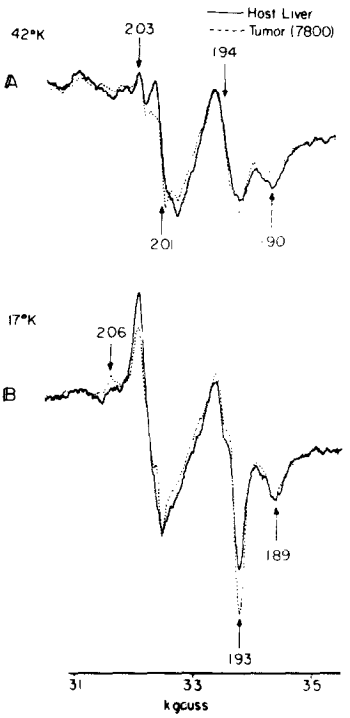

Figure 3. EPR spectra of reduced iron-sulfur centers in mitochondria prepared from Morris hepatoma 7800 and host liver, measured at 42° K and 17° K. Mitochondria used in this experiment were stored frozen in liquid nitrogen and thawed just before making samples for EPR measurement. Thus, anaerobiosis was obtained by incubating the mitochondrial suspension with 8.3 mM glutamate plus 8.3 mM malate and 3.3 mM NADH, protein concentrations of both hepatoma 7800 and host liver mitochondria were 50.0 mg/ml. EPR operating conditions were the same as described in the legend of Fig. 1, except for the temperature of spectrum A.

sulfur center N-1 (g_1 = 2.03, g_2 = 1.94) and center S-1 (g_1 = 2.03, g_2 = 1.94, and g_3 = 1.92), and signals from Rieske's Fe-S center (g_1 = 2.03, g_2 = 1.90, g_3 = 1.80) are observed at 58° K. When the temperature of the EPR measurement was lowered to 17° K, signals from iron-sulfur center N-2 (g_1 = 2.06 and g_2 = 1.93) and center 5 (g_1 = 2.09 and g_2 = 1.89) were observed. The peak position and line shape of these EPR signals arising from different iron-sulfur centers are similar to those observed in pigeon heart mitochondria (3). As shown by the dotted line of spectrum A, the line shape of individual Fe-S centers remained almost unaltered. Peak to peak amplitude of "g = 1.94" signal showed about 30% decrease in hepatoma 16 mitochondria. Because of the almost unchanged signal height at g = 1.92, iron-sulfur center S-1 seems to have no significant decrease in hepatoma 16 mitochondria. If it is assumed that about 50% of "g = 1.94" signal arises from center S-1 and the rest from center N-1, 30% overall decrease of "g = 1.94" amplitude corresponds to about 60% decrease

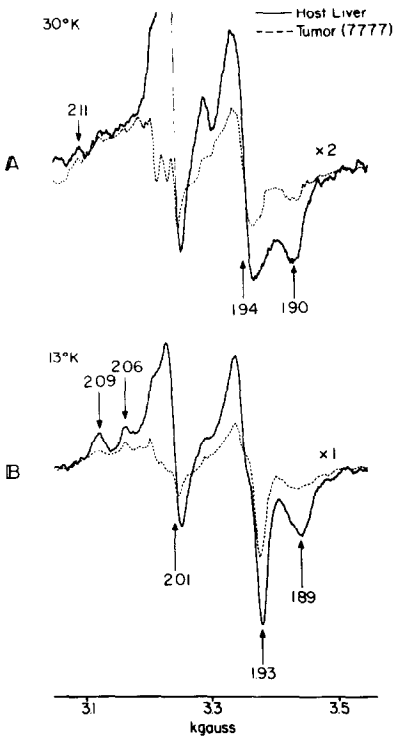

Figure 4. EPR spectra of reduced iron-sulfur centers in mitochondria prepared from rapidly growing Morris hepatoma 7777 and host livers, measured at 30° K and 13° K. Mitochondrial suspensions were treated as described in the legend of Fig. 1. Protein concentration of tumor and host liver mitochondria were 62.5 mg/ml and 65 mg/ml, respectively. EPR operating conditions are the same as those in Fig. 1.

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of center N-1 signal. At lower temperatures a dramatically diminished signal of center N-2 was observed from both g = 2.06 and 1.93 signals. Although spectra are not shown here, EPR signals arising from iron-sulfur center N-3 plus N-4 also showed a diminished intensity as great as in the case of center N-1. In contrast to iron-sulfur centers N-1, N-2 and N-3 plus N-4, EPR signals due to iron-sulfur centers 5, S-1 and Rieske's Fe-S center show only slightly diminished signals. These results indicate that in slow growing Morris hepatoma 16 mitochondria, iron-sulfur centers associated with the NADH dehydrogenase region of the respiratory chain are specifically diminished. This effect is reflected in the most decreased respiratory rate observed in mitochondria incubated with NAD-linked substrates compared with succinate or ascorbate plus TMPD.

Spectra A and B in Fig. 3 illustrate that the intensity of EPR signals from different iron-sulfur centers in mitochondria from Morris hepatoma 7800 of intermediate growth rate is almost unchanged from those observed with host liver mitochondria. This finding is also in agreement with the relative

respiratory rates observed with different substrates, which showed no significant differences from those of the host liver mitochondria.

In Fig. 4, EPR spectra of different iron-sulfur centers in the rapidly growing Morris hepatoma 7777 and host liver mitochondria are presented. As seen in spectrum A for the host liver mitochondria, EPR signals from iron-sulfur center 6 at g = 1.90 are partly overlapped with signals from centers 5 and Rieske's Fe-S center having a trough at g = 1.89, and the signals from centers N-1 and S-1 are overlapped at g = 1.94. All signals are greatly diminished compared to those in host liver mitochondria. At lower temperatures (spectrum B), centers N-1, N-2 and center 5 signals were obtained similar to spectrum B in Fig. 2. Again, all of the EPR signals from these iron-sulfur centers are diminished in mitochondria prepared from this rapidly growing tumor. These results also agree with the reduced respiration rates observed with all substrates.

DISCUSSION

These results demonstrate a remarkable correlation between the relative intensity of EPR signals arising from various iron-sulfur centers and the electron transfer rates in different spans of the respiratory chain, as indicated by respiration rates with various substrates which provide electrons that enter the respiratory chain at different sites. All iron-sulfur centers on the NADH side of the rotenone block showed diminished EPR signals in tumor 16. The disappearance of center N-2 signals was especially dramatic in a wide range of temperature and power settings of EPR measurements. In contrast to specifically diminished EPR signals of iron-sulfur centers in the Site I region, iron-sulfur centers in the Site II region and cytochrome b and $a+a_3$ showed only slight decrease, and cytochrome c exhibited a tendency to increase in this slow growing tumor[1], in agreement with observations by Hagihara et al. (25). Thus, the decrease of EPR signals at Site I region seems to be associated with the decreased respiration with NAD-linked substrates. State 3 respiration rates of these mitochondria metabolizing α-ketoglutarate (39.2 μatoms oxygen/min/mg protein), pyruvate plus malate (36.8 μatoms/min/mg protein), and palmitylcarnitine plus malate (36.3 μatoms/min/mg protein) were very similar to rates obtained with glutamate plus malate (38.0 μatoms/min/mg protein). This similarity may indicate that the rate-limiting step is in the NADH dehydrogenase region of the electron transport chain rather than in the NAD-linked substrate dehydrogenases. The rapidly growing hepatoma 7777 exhibited diminished respiratory rates with all substrates tested, and it

[1] J.G. Hemington, unpublished observations.

correlated with diminished EPR signals of all iron-sulfur centers. This correlation also holds with changes in cytochromes; cytochromes b and $a+a_3$ decreased to about 50% of the concentration in host liver mitochondria (cf. refs. 20,26). However, the important observation about this rapidly growing tumor mitochondria is that the decrease of EPR signal from iron-sulfur center N-2 (Fig. 3, spectrum B) is not so prominent as that in center N-2 of slow growing hepatoma 16 (Fig. 2, spectrum B). This relates very well with a less prominent decrease of respiratory rate with NAD-linked substrates in hepatoma 7777, in comparison with that in hepatoma 16.

Preliminary studies of tissue slices from tumor 16 indicate that this hepatoma is characterized by higher lactate production rates than host liver slices during aerobic glycolysis. It is possible to suggest that the abnormal metabolic characteristics of some tumors described in the literature (23,27) may result from a specific defect in the iron-sulfur components of the electron transport chain, as indicated in hepatoma 16.

There is no general relationship between the defect in respiratory chain components observed in this series of hepatoma mitochondria and the observed growth rates or the reported generation times for the tumors (27). As reported in the present communication, hepatoma 7800 mitochondria apparently have a normal electron transport chain which is intermediate in growth rate. Of interest in this regard is the report by Aisenberg and Morris (28) that hepatoma 7800 displayed no aerobic or anaerobic glycolysis, no Crabtree effect, good respiratory response to succinate and was metabolically uncharacteristic of tumors.

Further generalizations relating the iron-sulfur proteins of the electron transport chain to metabolic characteristics of tumors require more detailed studies on a wider range of different types of tumors. Additional information on the functional role of iron-sulfur centers in mitochondria may provide further insight into the defects of metabolism in other tumors.

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