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THE ELECTRON PARAMAGNETIC RESONANCE OF MOLYBDENUM IN RAT LIVER AND IN RAT LIVER MITOCHONDRIA

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SUMMARY

1. A spectral feature of the EPR spectrum of rat liver and rat liver mitochondria observed near $g = 1.96$ arises from a paramagnetic form of molybdenum. Liver mitochondria prepared from untreated control animals, from animals treated with ^{92}Mo ($I = 0$) or from animals treated with the naturally occurring mixture of molybdenum isotopes show a doublet in the $g = 1.96$ region while liver mitochondria from rats treated with ^{95}Mo ($I = 5/2$) show no such EPR absorptions.

2. Resuspension of mitochondria from control animals in sucrose-^2H_2O solution converts the EPR doublet to a singlet suggesting interaction of paramagnetic molybdenum with protons.

3. The addition of NADH to rat liver mitochondria which had been frozen diminishes the resonance ascribable to molybdenum.

4. It is believed that the EPR of molybdenum in rat liver mitochondria, which cannot be observed under the same conditions in beef heart mitochondria, arises from molybdoproteins previously isolated from liver and not from proteins involved in mitochondrial electron transport.

INTRODUCTION

Recent interest in the possible role of molybdenum (Mo) in mitochondrial electron transport has generated new EPR studies of mammalian mitochondria and submitochondrial particles[1]. For these preparations it is well known that the EPR spectrum in the region near $g = 2$ is rather complex, containing features arising from various paramagnetic components of electron transport including free radical species[2–5], copper of cytochrome oxidase[6,7], non-heme iron proteins[2], as well as iron in cubic fields which is a common contaminant. More recently, DERVARTANIAN AND BRAMLETT[8] showed that chemically reduced NADH dehydrogenase, isolated from *Azotobacter vinelandii* which had been grown in an iron deficient medium containing Mo, indeed had the EPR characteristics of Mo (V), with assigned g values of 2.018, 1.977 and 1.948. More recent studies by ALBRACHT AND SLATER[1] of Complex I of beef heart mitochondrial electron transport (NADH ubiquinone reductase) showed

similar EPR signals in this preparation ($g = 2.00$, 1.97 and 1.95) but these signals appeared only after long incubation at room temperature in the presence of NADH. Although the presence of Mo in the preparations used by Slater and Albracht was determined by atomic absorption spectroscopy, it is clear from their study that other paramagnetic species were in the sample and that the EPR assignment they give to Mo is equivocal.

This present paper describes experiments which do show that the EPR resonance near $g = 1.96$ indeed arises from molybdenum in isolated rat liver mitochondria. Taking advantage of the fact that ^{92}Mo has a nuclear spin of zero while ^{95}Mo has a nuclear spin of five halves, and that the EPR of these two species is therefore different, we have exchanged the molybdenum pool in rats using two stable isotopes, ^{92}Mo and ^{95}Mo. We have examined the EPR spectra of liver slices as well as of isolated mitochondria from animals treated with different Mo isotopes in a manner analogous to the study of xanthine oxidase by BRAY AND MERRIWEATHER[9].

MATERIALS AND METHODS

Molybdenum metal powder, containing a naturally occurring mixture of isotopes, was purchased from Alpha Inorganic. ^{92}Mo (98.3 atomic percent) and ^{95}Mo (96.4 atomic percent) metal powders were obtained from Oak Ridge National Laboratories. Each metal sample was dissolved separately in *aqua regia*, which was then gently boiled to dryness. The resulting white powders were dissolved in dilute NH_3-water and the pH was adjusted to 7–8 with dilute acetic acid. These three solutions were each diluted so as to contain 1 mg/ml Mo in a soluble form.

Three groups of female Wistar rats, each weighing approx. 75 g, were maintained with *ad libitum* feeding of low Mo test diet (Nutritional Biochemical) and distilled water. After two weeks, each of the rats in the three groups was injected with the respective Mo solution, while the drinking water was adjusted to contain the same molybdenum solution used for injection, but at a concentration of 1 mg/l. After the initial injection, further injections of 0.5 ml of the same Mo solutions used previously were repeated every three days over a period of twelve days. At this time, the animals were decapitated and liver samples from at least two rats in any single group were washed in cold water followed by iced 0.25 M sucrose solution, blotted on filter paper, weighed, and quickly frozen in EPR cavities within 3 min after decapitation of the animal. These samples were maintained at liquid N_2 temperature (77° K) until the EPR spectra were examined. The remainder of the livers, together with the liver from a third animal, was homogenized in an all glass Dounce homogenizer in 0.25 M sucrose solution, and mitochondria were prepared by the method of SCHNEIDER AND HOGEBOOM[10]. The mitochondrial fractions were resuspended in sucrose solution and centrifuged at 10000 × g for 10 min in order to remove organelle contaminants. Mitochondrial preparations obtained from each of the three groups of Mo-treated animals were frozen in EPR cavities and maintained at 77°K until they were used for EPR examination. The mitochondrial preparations contained no detectable microsomes since the EPR of mammalian cytochrome P-450[11] was absent in all of them.

A group of control rats, which were maintained in the same animal quarters, were not injected with Mo solutions and were fed *ad libitum* with Purina rat chow diet while tap water was made available to them. Liver samples and mitochondrial prep-

arations from these animals were also obtained and treated identically with those described above.

A sample of mitochondria from untreated control animals was suspended three times with 30 vol. of 0.25 M sucrose solution prepared in ^2H_2O. Each time after dilution, the mitochondria were centrifuged at 10000 $\times$ *g* for 10 min in capped centrifuge tubes, and the pellet of the last centrifugation was frozen in an EPR cavity.

EPR spectra were taken on a superheterodyne spectrometer[12], to which a Varian Fieldial was attached, operating near 9200 Mcycles/sec at a temperature of 1.4°K. Fieldial calibration was performed with a proton magnetic resonance probe. The samples contained about 0.8 g wet wt. of liver slices or of mitochondrial pellet.

RESULTS

The EPR spectra in the region near $g = 2$ for liver slices and for liver mitochondria from Mo treated rats and from untreated control rats are shown in Fig. 1. As can clearly be seen, liver slices (Figs. 1A, 1E) and mitochondria from untreated control animals or animals treated with ^{92}Mo (Fig. 1B) exhibit absorptions near $g = 1.96$ which are absent in mitochondria (Fig. 1D) and liver slices (Fig. 1C) from ^{95}Mo-treated animals. At lower field modulation and low spectrometer power ($1 \cdot 10^{-8}$ W) the absorption derivative near $g = 1.96$ resolves into a doublet with measured *g* values (as absorption derivative minima) of 1.969 and 1.961 and a separation of 13 gauss. Those animals treated with the naturally occurring Mo isotopic mixture also showed the same resonances near $g = 1.96$. If these EPR spectra have another *g* value to higher field, then these resonances should be measured at the zero crossing, and thus the *g* values would be slightly larger. It is noteworthy that the intensity of the EPR signals near $g = 1.96$ for liver slices from untreated animals is about 20% less per weight of liver than for liver slices from ^{92}Mo-treated animals.

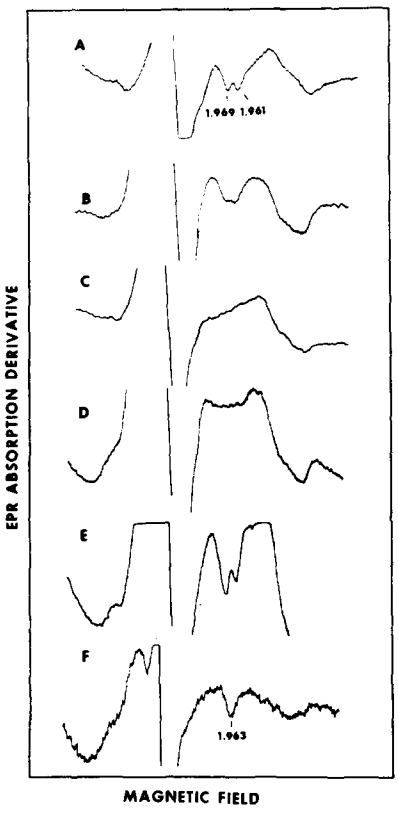
Mitochondria which were repeatedly suspended in sucrose–^2H_2O and then examined with EPR no longer showed the doublet near $g = 1.96$ but instead showed a singlet centered at $g = 1.963$ (Fig. 1F). Reducing the field modulation of the spectrometer to 2.5 Gauss did not further resolve the singlet at $g = 1.963$, but a small absorption near $g = 1.973$ could be seen at higher gain. The integrated area of the $g = 1.963$ absorption is approximately equal to the sums of integrated areas of the doublet observed in mitochondria before the repeated sucrose–^2H_2O resuspension.

Liver mitochondria from untreated control animals or from those animals treated with the naturally occurring isotopic mixture of Mo or with ^{92}Mo were treated with NADH (Fig. 2). As can be seen, the EPR intensity of the $g = 1.96$ resonance decreases significantly after NADH treatment, suggesting interaction of paramagnetic material with reduced pyridine nucleotide.

DISCUSSION

Molybdenum as a metallic component has been observed in the purified mammalian enzymes xanthine oxidase[9], xanthine dehydrogenase[13], aldehyde oxidase[14] and sulfite oxidase[15]. In all proteins, the paramagnetic form of Mo is believed to be Mo (V) in which the paramagnetism is due to a single unpaired *d* electron ($4d^1$). The EPR of this material can best be described by three *g* values, two less than 2 and the third equal

to or greater than 2. A naturally occurring isotopic mixture of Mo contains 75% of material having a nuclear spin of zero as does ^{92}Mo, and 25% of material having a nuclear spin of five-halves, as does ^{95}Mo. The EPR of the material with the high nuclear spin has a more complex spectrum with six hyperfine lines for each g value.



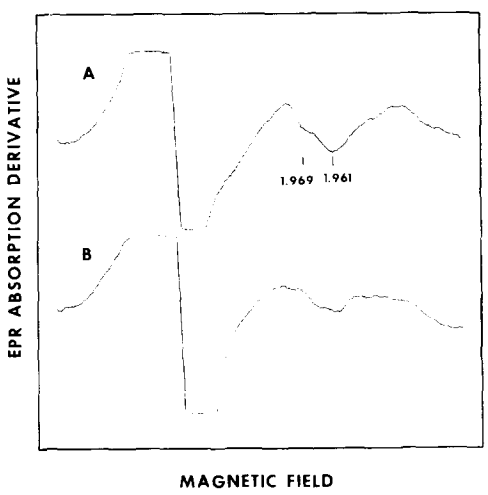


Fig. 1. EPR spectra of rat liver slices and rat liver mitochondria in the region near $g = 2$ for ^{92}Mo- and ^{95}Mo-treated animals. A. Rat liver slices from ^{92}Mo-treated animals. B. Rat liver mitochondrial pellet from ^{92}Mo-treated animals. C. Rat liver slices from ^{95}Mo-treated animals. D. Rat liver mitochondria from ^{95}Mo-treated animals. E. Rat liver slices from untreated control animals. F. Rat liver mitochondria from control animal after $^{2}H_2O$ exchange. All spectra were taken at a sweep rate of 200 Gauss/min. The spectrometer gain was the same for all traces except E which was taken at twice the gain of the others. The spectrometer was operated at 5 Gauss modulation (A and C) or at 7 Gauss modulation (B, D and E) with a time constant of 0.3 sec for all traces. Modulations greater than 7 Gauss significantly broadened the doublet resonance near $g = 1.96$ into a singlet while spectral examination at 2.5 Gauss modulation did not show any increased narrowing. The EPR features near $g = 1.96$ saturated very readily and the spectra shown here, which were not significantly saturated, were taken at $1 \cdot 10^{-8}$ W, the optimum operating power of the spectrometer for these samples.

Fig. 2. EPR spectra showing the effect of NADH on rat liver mitochondria from untreated animals. A. Rat liver mitochondria. The EPR of this material does not change upon thawing and refreezing. B. The mitochondria were thawed after spectroscopic examination and 0.1 ml of 1 mM NADH was added. Then the mitochondrial suspension in the EPR cavity was stirred for 30 sec and quickly refrozen (30 sec) in liquid N_2. Spectra were taken with a field modulation of 7 Gauss, a time constant of 1 sec and a sweep rate of 100 Gauss/min. The loss of resolution of the $g = 1.96$ doublet is caused by difference in time constant from that of Fig. 1.

Although the area of the integrated form of the EPR absorption derivative for material with zero nuclear spin is the same as for material with spin five-halves, the EPR spectrum for the latter paramagnetic Mo isotope will consist of eighteen features spread over a greater range of magnetic field. Thus sharp EPR absorption derived from ^{92}Mo will appear as many small absorptions for ^{95}Mo.

In the case of rat liver slices and liver mitochondria the EPR features observed for the ^{92}Mo-treated animals can no longer be seen for the ^{95}Mo-treated animals since the EPR intensity is now spread into many hyperfine lines, having much smaller intensity, which were not detectable at the signal-to-noise level of these experiments. Thus, the region near $g = 1.96$ shows no EPR absorptions. Furthermore, the lack of signal near $g = 1.96$ in rat liver mitochondria from ^{95}Mo-treated animals suggests that all or nearly all of the naturally occurring Mo has been exchanged with ^{95}Mo by the treatment described above. Also, the signal intensity at $g = 1.96$ for liver preparations from untreated animals is only slightly less than for ^{92}Mo-treated animals and is the same as for preparations from rats treated with the natural isotopic mixture. This leads us to believe that a paramagnetic form of Mo is not unspecifically absorbed by liver and liver mitochondria as a result of the loading dosage of Mo given since the EPR signal intensity near $g = 1.96$ would probably be greater in these cases.

Under certain conditions, the separation between g_x and g_y for Mo (V) of aldehyde oxidase when observed at x-band (near $g = 1.972$) is only about 11 Gauss[16]. For xanthine oxidase[9, 17], in the presence of substrate, the separation between g_x and g_y is even smaller. In the signal that we ascribe to Mo in our rat liver preparations, it might be thought that the doublet that we see represents the splitting of g_x and g_y. However, the ^2H_2O exchanged material no longer shows this splitting, and in fact, the doublet collapses into a singlet absorption at $g = 1.963$ at a position in field between those for the doublet. Similar findings were observed for purified sulfite oxidase which was exchanged with ^2H_2O[15]. This leads us then, to the conclusion that the resonance that we see at $g = 1.969$ and $g = 1.961$ represents a single feature of an Mo (V) spectrum, where the splitting is due to interaction of the electron spin of Mo (V) with a single proton. Protonic splitting of a Mo resonance of from 12 to 14 Gauss has been reported for xanthine oxidase in the presence of substrate[17]. The other features of an EPR spectrum for Mo (V) in mitochondria are most likely to lower field where they are masked by EPR absorptions from other paramagnetic species.

The question now raised is what is the biological significance of Mo in rat liver mitochondria? It is quite clear that NADH can partially reduce paramagnetic Mo in rat liver mitochondria that had been frozen. Yet, our preliminary experiments on beef heart mitochondria do not show the resonance which we believe arises from Mo (V) in liver preparations. It is hard to believe that the role of Mo in electron transport in two different tissues should vary, and it is further more unlikely that this variation is due to species differences.

A different explanation which we favor is that the EPR we observe near $g = 1.96$ which arises from a paramagnetic form of Mo (V) is that of one or more Mo-containing proteins which have been previously isolated from liver. Two of these, aldehyde oxidase[14] and sulfite oxidase[15], have EPR features with g values in the region of those that we ascribe to Mo in rat liver mitochondria. Still, the question whether

EPR detectable Mo is a component of electron transport in rat liver mitochondria has not been completely resolved. An approach that can be taken is to determine the change of intensity of the EPR near $g = 1.96$ during electron flow[14] and to correlate these findings with the kinetics of electron transport studied optically[18]. The inherent difficulty of such an experiment is due to the limits of the EPR technique. The $g = 1.96$ resonance observed by us in rat liver mitochondria is extremely weak and furthermore, lies in a region of magnetic field containing resonances from many paramagnetic species. A significant improvement in the EPR spectrometer signal to noise ratio may make such experiments tenable and the question concerning the role of Mo in mitochondrial electron transport can then be settled.

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REFERENCES

1 S. P. ALBRACHT AND E. C. SLATER, *Biochim. Biophys. Acta*, 223 (1970) 457.
2 H. BEINERT AND W. LEE, *Biochem. Biophys. Res. Commun.*, 5 (1961) 40.
3 H. BEINERT, W. HEINEN AND G. PALMER, *Brookhaven Symp. Biol.*, 15 (1962) 229.
4 Y. HATEFI, A. G. HAAVIK AND D. E. GRIFFITHS, *J. Biol. Chem.*, 237 (1962) 1676.
5 H. BEINERT AND R. H. SANDS, *Biochem. Biophys. Res. Commun.*, 3 (1960) 41.
6 R. H. SANDS AND H. BEINERT, *Biochem. Biophys. Res. Commun.*, 1 (1959) 175.
7 H. BEINERT, D. E. GRIFFITH, D. C. WHARTON AND R. H. SANDS, *J. Biol. Chem.*, 237 (1962) 2337.
8 D. V. DERVARTANIAN AND R. BRAMLETT, *Biochim. Biophys. Acta*, 220 (1971) 443.
9 R. C. BRAY AND C. S. MERRIWEATHER, *Nature*, 506 (1966) 467.
10 W. C. SCHNEIDER AND G. H. HOGEBOOM, *J. Biol. Chem.*, 183 (1950) 123.
11 J. PEISACH AND W. E. BLUMBERG, *Proc. Natl. Acad. Sci. U.S.*, 67 (1970) 172.
12 J. PEISACH, W. E. BLUMBERG, E. A. RACHMILEWITZ, S. OGAWA AND R. OLTZIK, *J. Biol. Chem.*, 246 (1971) 3342.
13 S. T. SMITH, K. V. RAJAGOPALAN AND P. HANDLER, *J. Biol. Chem.*, 242 (1967) 4108.
14 K. V. RAJAGOPALAN, P. HANDLER, G. PALMER AND H. BEINERT, *J. Biol. Chem.*, 243 (1968) 3784.
15 H. J. COHEN, I. FRIDOVICH AND K. V. RAJAGOPALAN, *J. Biol. Chem.*, 246 (1971) 374.
16 H. BEINERT AND G. PALMER, *Adv. Enzymol.*, 27 (1965) 105.
17 R. C. BRAY AND T. VÄNNGÅRD, *Biochem. J.*, 114 (1969) 725.
18 B. CHANCE, B. SCHOENER AND D. DEVAULT, in T. E. KING, H. S. MASON AND M. MORRISON, *Oxidases and Related Redox Systems*, Wiley, New York, 1965, p. 907.