A COMPLEX EPR SIGNAL IN MUNG BEAN MITOCHONDRIA AND ITS POSSIBLE RELATION TO THE ALTERNATE PATHWAY

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SUMMARY

Low temperature EPR investigations of mung bean mitochondria in various respiratory states have revealed a complex signal split around g = 2.00. The signal is paramagnetic in the partially oxidized state and under conditions in which an alternate pathway to molecular oxygen is operative. The signal is absent from mung bean mitochondria respiring in State 3 but can be observed in State 4 or in the presence of cyanide. The signal is not observed in the presence of SHAM, an inhibitor of the alternate pathway. It is proposed that the observed spectrum may be related to, but more complex than, that observed previously in animal mitochondria, which has been interpreted as a spin-spin interaction between two ubisemiquinones relaxed by Center S-3.

INTRODUCTION

Extensive literature is available on the iron-sulfur centers of mammalian mitochondria [see review (1)]. Recently a class of iron-sulfur centers was reported to exist in mammalian mitochondrial preparations which are paramagnetic in their oxidized forms (2, 3, 4), as are the bacterial high potential iron-sulfur proteins (5). It has been shown that intact mammalian mitochondria contain two different 'Hipip'-type species, these species being distinguised by their temperature and microwave power saturation profiles and by the absence of one of the species from sub-mitochondrial particle preparations (4).

It is well established that some plant mitochondria possess an alternate pathway to molecular oxygen which is insensitive to antimycin A and cyanide (see reviews 6, 7). Relatively little information, however, is available on the iron-sulfur proteins of plant mitochondria or in particular upon their role in cyanide-insensitive respiration. Their presence on the alternate pathway

was suggested by the selective inhibition of the alternate respiratory pathway by metallo-chelating agents (8), by hydroxamic acids (9) or by piericidin A or 2 thenoyltrifluorooacetone (10). Moreover Bendall and Bonner (11) found that sub-mitochondrial particles of Symplocarpus foetidus behaved towards cyanide and hydroxamic acids in the same way as intact mitochondria, giving a large EPR signal at g = 1.92 when reduced by dithionite. In anaerobic skunk cabbage particles an EPR signal near g = 2 and g = 1.94 was enhanced by saturating concentrations of m-iodobenzhydroxamic acid, especially in the region g = 2 (9). This signal was attributed to an unknown iron-sulfur protein in the alternate pathway. During the induction of cyanide-insensitive respiration in potato or Jerusalem artichoke tissue, Cammack and Palmer (12) noted a slight decrease in the level of the iron-sulfur centers of NADH-ubiquinone reductase but no new signal due to the progressive presence of the alternate pathway could be detected. However in submitochondrial particles of Arum maculatum, reduced by dithionite, an intense signal at g = 2.02 and 1.93 was detected and since this was consistent with a very high level of iron-sulfur center N-1, it was suggested that this center might be involved in the alternate pathway of Arum mitochondria (12). A similar suggestion was made by Jensel et al (13) proposing that the branchpoint for the alternate pathway in submitochondrial particles of a respiratory mutant of Neurospora crassa occurs before iron-sulfur center N-2.

In view of the possible interaction of Hipip type iron-sulfur centers with the semiquinone forms of ubiquinone in mammalian mitochondria (14, 15, 16) and the recent proposal from this laboratory that the alternate pathway reverses the $QH^{\bullet}/QH_2$ couple of succinate dehydrogenase (17), it was considered necessary to investigate the Hipips of plant mitochondria and their possible relation to the alternate pathway. This communication provides evidence for at least one type of Hipip type iron-sulfur center in mung bean mitochondria, which under certain conditions is accompanied by partially overlapping signals, on the high and low field side. These signals are absent from mitochondria respiring in State 3 or in the presence of salicyl-hydroxamic acid but can be observed in mitochondria

respiring in State 4 or in the presence of cytochrome oxidase inhibitors. A preliminary account of the results of this investigation has been presented orally (18).

MATERIALS AND METHODS

Etiolated mung bean hypocotyls (Phaseolus aureus) were grown for 5 days in a dark room maintained at 28° and 60% relative humidity. Mitochondria were prepared as described by Bonner (19). Samples for EPR measurements of mitochondria in various metabolic states were frozen rapidly in an isopentane-cyclohexane freezing mixture (81K) and stored in liquid nitrogen until assayed.

All spectra were obtained on a Varian E-4 EPR spectrometer (Varian Associates) The temperature of the samples for EPR measurements were controlled using a variable temperature cryostat (Air Products Model LTD-3-110). Temperature measurement and g-value corrections were conducted as described by Ohnishi (20). Quartz glass EPR sample tubes were calibrated with a standard copper sulphate-EDTA solution and values for signal heights were corrected correspondingly.

All standard chemicals used were of the highest grade available commerically. Protein was measured by the method of Lowry et al (21).

RESULTS

Low temperature EPR investigations of mung bean mitochondria have revealed a signal split around g = 2.00 which is paramagnetic in the partially oxidized or reduced states. Mitochondria treated with 10 mM succinate as substrate, a condition under which the alternate pathway is operative (22), reveal a complex spectrum with peaks at g = 2.046, 2.033, 2.017, 1.986, 1.969 and 1.960 (Fig. 1). The signal centered at g = 2.01 and having a maximum at g = 2.017 is comparable

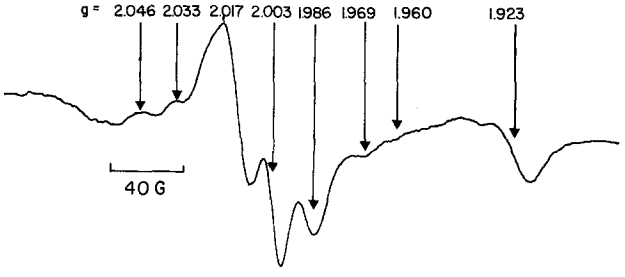

Figure 1. EPR spectrum of mung bean mitochondria in State 4. Spectra were recorded at 12K at the following instrument settings: field modulation frequency, 100 kHz; microwave power, 5 mW; microwave frequency, 9.13 GHz, modulation amplitude, 12.5 gauss; time constant, 1 sec; scanning rate, 400 gauss/min. Mung bean mitochondria (33 mg protein/ml) were suspended in a medium containing 300 mM mannitol, 5 mM $MgCl_2$, 10 mM KCl and 10 mM potassium phosphate pH 7.4.

to the Hipip signal found in mammalian mitochondria (4) under similar conditions and is identified as Center S-3,an iron-sulfur center associated with succinate dehydrogenase.

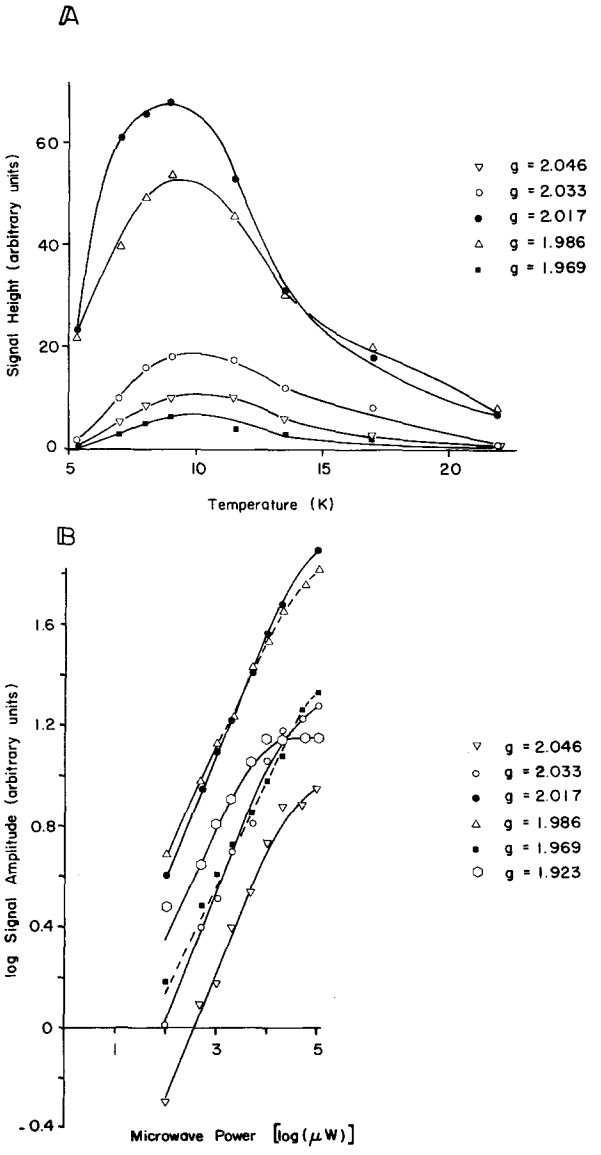

Figure 2. Temperature and power dependence of the EPR signals examined in mung bean mitochondria. Mung bean mitochondria were suspended to a final concentration of 33 mg/ml in a media identical to that described in Figure 1. In A, the EPR sample temeprature was varied; the microwave power was 5 mW. In B, the temperature was 12K and the input microwave power was varied. The instrument settings were: microwave frequency, 9.13 GHz; modulation amplitude, 12.5 gauss; time constant, 1 sec; scanning rate, 400 gauss/min.

Figure 2 shows the temperature and microwave power dependencies of this spectrum. The temperature depedencies of the g = 2.046, 2.033, 2.017, 1.986, and 1.969 signals are plotted in Figure 2A as the absolute temperature against signal height. The relative signal height was determined as a peak height from a low magnetic field base line. It is apparent from Figure 2A that these signals can be detected in mung bean mitochondria over a similar range of temperatures. These signals show approximately similar temperature profiles. The microwave power dependencies are illustrated in Figure 2B. Since they all saturate at the same power level (≃10 mW) at given temperatures, the species giving rise to these signals must have similar relaxation rates as has been shown to be the case in animal systems (3, 16). Also included in Figure 2B is the power dependency of the g = 1.923 signal (not included in 2A). It is readily apparent that this signal does not have the same saturation level as the other five signals and is therefore not part of the same species. The g = 1.923 signal is possibly due to Center N-2, a center associated with the NADH dehydrogenase, which is paramagnetic in the reduced form [in contrast with Hipip species (1)] and the $g_{\parallel}$ -value corresponding to the $g_{\perp}$ = 1.923 is small and broader than its equivalent and is at g = 2.05.

Figure 3 presents EPR spectra obtained with mung bean mitochondria in various respiratory states recorded under EPR conditions optimal for the six signals. In order to obtain a maximally oxidized state of the Hipip signal, spectrum Fig. 3 (g) was obtained with mitochondria oxidized with 500 μM ferricyanide in the absence of added substrate. Spectra in Fig. 3 (a)-(f) were obtained from mitochondria with added succinate as substrate. On addition of an artificial oxidant such as ferricyanide, signals comparable to those observed under State 4 conditions (Figs. 1 and 3e) are discernible. In the uncoupled and aerobic state (Fig. 3d) a condition in which electron flux through the alternate pathway is negligible (22), Center S-3 is largely oxidized as are other respiratory chain carriers in the region of loop two (23). Under these conditions the additional signals at g = 2.046, 2.033, 1.986, 1.969 and 1.960 are not observed.

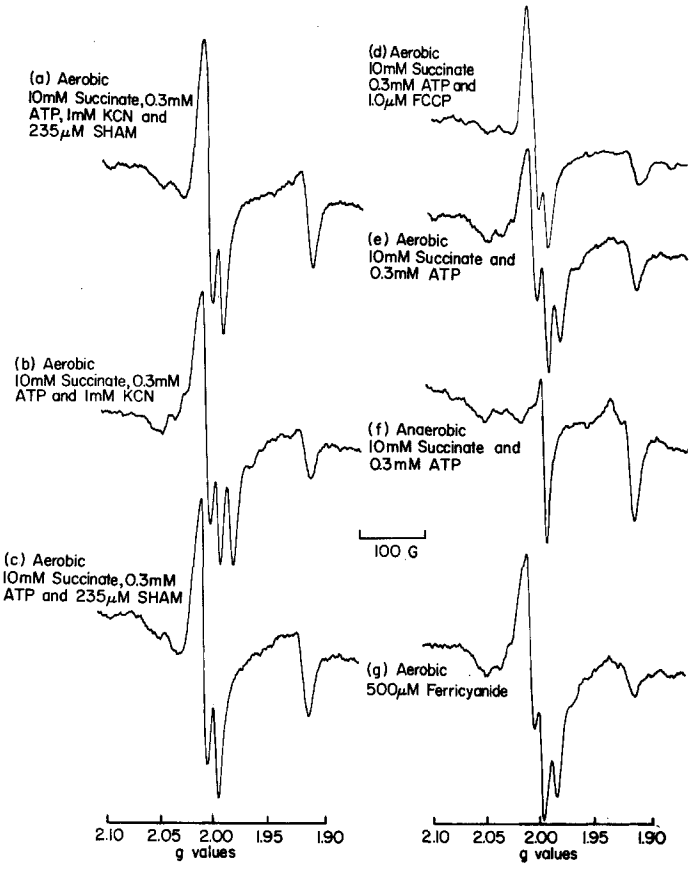

Figure 3. EPR spectra of mung bean mitochondria in different metabolic states, measured at 12K. Mung bean mitochondria were suspended to a final volume of 26 mg/ml in the reaction media as indicated in Figure 1. Concentrations of additions are as indicated. EPR conditions were similar to those used in Figure 1.

Figure 3 (b) reveals that the addition of 1 mM cyanide to mitochondria under State 4 conditions did not reduce Center S-3, as is the case in mammalian preparations (4) but enhanced all of the signals (increase in signal height). On addition of SHAM, a specific inhibitor of the alternate pathway (9) (Figure 3a, c) to mitochondria either under State 4 conditions or in the presence of cyanide, the additional signals at g = 2.046, etc., disappear and a spectrum similar to that under State 3 conditions (Fig. 3d) is observed. Upon anaerobiosis Center S-3 and the additional signals are reduced whereas the g = 2.003 (free radical) and g = 1.923 (N-2) signals are enhanced.

DISCUSSION

The present investigation provides evidence for the existence of at least one Hipip type iron-sulfur center in mung bean mitochondria which is identified as Center S-3 of succinate dehydrogenase. Under certain conditions, Center S-3 is accompanied by partially overlapping signals at g = 2.046, 2.033, 1.986, 1.969 and 1.960.

To determine whether Center S-3 and the overlapping signals function in the respiratory chain, and in particular are observed during alternate pathway activity, their appearance under various metabolic conditions was studied. Although the response of Center S-3 and the overlapping signals indicated a relationship between the signals and the respiratory chain; it appears to be more complex than its counterpart in mammalian systems. For example, in State 4, a condition under which Center S-3 in mammalian mitochondria is 60% reduced (4), in mung bean mitochondria it is largely oxidized (Fig. 3e). Similarly, the addition of cyanide did not reduce Center S-3 or the overlapping signals as is the case with mammalian systems (4). Of special significance was the finding that SHAM caused the loss of the signals at g = 2.046, 2.033, 1.986, 1.969 and 1.960 without changing Center S-3 or the free radical signal. This is a particularly interesting observation, since in the presence of cyanide, electron flux through the alternate pathway is maximal (22) and is specifically inhibited by SHAM. The effect of cyanide and SHAM on the oxidation state of Center S-3 and the overlapping signals allows speculation concerning the possible role of the species responsible for the additional absorbances in mediating electron transport via the alternate pathway. We are currently examining this point in further detail.

It has recently been shown that mammalian Center S-3 is accompanied by similar partially overlapping signals at g = 2.04, g = 1.99 and g = 1.96 under certain conditions (14, 3) ubisemiquinone being involved in a dipole-dipole interaction giving rise to these signals (14). More specifically these additional absorbances have been ascribed to a spin-spin interaction between ubisemiquinones with Center S-3 acting as a magnetic relaxer (16). Recent

evidence suggests that both Center S-3 and the species giving rise to the g = 2.04, 1.99 and 1.96 absorptions are functional members of the respiratory chain (15, 16). It is therefore possible that although the overlapping signals observed in these experiments may be attributed to a spin-spin interaction of a paramagnetic species, involving Center S-3 and ubisemiquinone, it is more complex than that previously observed in mammalian preparations. It may be concluded that the apparent requirement of Center S-3 for the observation of the overlapping signals (14, 3, 16 and present) and the location of this Center on the matrix side of the mitochondrial membrane (4) further supports the suggestion (17) that the specific location of the branchpoint of electron flow into the alternate pathway is at the reversal of the step of succinate dehydrogenase reduction of QH• to QH_2. The interaction of the succinate dehydrogenase with the ubiquinone pool has recently been shown to involve the QH•/QH_2 couple (15, 16).

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