Biochimica et Biophysica Acta, 504 (1978) 345—363

BBA 47575

AN EPR ANALYSIS OF CYANIDE-RESISTANT MITOCHONDRIA ISOLATED FROM THE MUTANT POKY STRAIN OF *NEUROSPORA CRASSA*

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(Received February 27th, 1978)

Summary

An analysis of the paramagnetic components present in mitochondria isolated from the poky mutant of *Neurospora crassa* is described. The study was undertaken with a view to shedding light on the nature of the cyanide- and antimycin A-resistant alternative terminal oxidase which is present in these preparations.

Of the ferredoxin-type iron-sulfur centers, only Centers S-1 and S-2 of succinate dehydrogenase could be detected in significant quantities. Paramagnetic centers attributable to Site I were virtually absent. In the oxidized state, at least two 'high potential iron sulfur' centers could be distinguished and these were attributed to Center S-3 of succinate dehydrogenase and a second component analogous to that found in mammalian systems. Much of the Center S-3 signal was in a highly distorted state which was apparently dependent upon the presence of an accompanying free radical species. At lower field positions, a succinate-reducible signal peaking around $g = 3.15$ was found. This signal is caused by a low spin heme species, presumably the cytochrome *c* which is the only major cytochrome in these mitochondria. At even lower field positions, signals attributable to iron in a field of low symmetry at $g = 4.3$ and multiple high spin heme species around $g = 6$, could be distinguished.

The effects of salicylhydroxamic acid, an inhibitor of the alternative oxidase, were tested on these components. Effects could be seen on at least one high spin heme component and also partially upon the distorted Center S-3 signal converting part of it to a signal indistinguishable from Center S-3. Some increase in the $g = 4.3$ iron signal was also noted. No effects of the inhibitor on the ferredoxin-type centers were detected.

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These results are interpreted with respect to the nature and location of the alternative oxidase and with respect to possible models for the nature of the alternative oxygen-consuming component.

Introduction

Mitochondria isolated from normally grown cells of *Neurospora crassa* are similar to mitochondria of mammalian, yeast and other systems in terms of cytochrome content, paramagnetic centers, phosphorylation sites, sensitivity to inhibitors and substrate oxidations [1—3]. When the cells are grown in the presence of chloramphenicol or antimycin A, or in mutant strains such as the poky mutant, however, the mitochondria have abnormal cytochrome contents and possess a rapid alternative respiratory pathway which is insensitive to cyanide and antimycin A [4—7]. This alternative pathway is analogous to that found in some higher plant mitochondria [8] on the basis that it is sensitive to hydroxamic acids [9,10] and branches from the main respiratory pathway at the level of ubiquinone [11].

We have recently studied the higher plant alternative pathway by the technique of EPR and have found that a part of the ubiquinone pool is the closest detectable component to the alternative oxygen-consuming step (ref. 12 and cf. ref. 13). Indeed, it has led us to propose that the autoxidisable component may be a pool of ubiquinone which is in a modified form (either by structure or environment) or which can be oxidized by an as yet EPR- and optically-invisible component [14]. Evidence for a site of interaction of salicylhydroxamic acid at or close to a ubisemiquinone pair which interacts with Center S-3 of succinate dehydrogenase was obtained although problems with the general lability of the signal and its sensitivity hydroxamic acids were encountered [12].

A comparison of the EPR properties of wild type and poky strains of *N. crassa* offers an excellent controlled system for the further study by EPR of the alternative oxidation step and the results of such an investigation are presented in this report. In the aerobic state, EPR signals characteristic of Center S-3 and a Ruzicka center were detected in isolated mitochondria of both wild-type and poky strains of *N. crassa*. Overlapping these components in both types of mitochondria was a small free radical $g = 2.00$ signal and a broad (approx. 80 gauss), rapidly relaxing signal, caused apparently by Center S-3 in a modified environment. Other components, detectable in aerobic samples, were found around $g = 3.15$ (low spin heme), at $g = 4.3$ (high spin iron in a field of low symmetry) and around $g = 6.0$ (high spin heme components).

The ferredoxin-type components of wild type *N. crassa* mitochondria have already been well-characterized by Warden and Edwards [3]. They presented evidence for the presence of Centers N-1 to N-5, the Rieske Center and a free radical species. Although not discussed, some contribution to their spectra by the ferredoxin-type centers of succinate dehydrogenase, Centers S-1 and S-2, must also have been present although these were severely masked by the other components. In poky mitochondria, however, it is shown in this report that the centers of Site I and the Rieske center are severely diminished in

content and that only Centers S-1 and S-2 remain in large amounts.

The effects of salicylhydroxamic acid on the EPR-detectable components have been tested, in the hope that this alternative oxidase inhibitor might perturb the signals of components close to or attributable to the alternative oxidase. Small effects were seen on at least one high spin heme signal, on the magnitude of the $g = 4.3$ iron signal, and upon the modified Center S-3 signal. These effects are most probably caused by properties of the hydroxamic acids which are secondary to their alternative oxidase-inhibiting capacity. It is concluded that the alternative oxidase is not an iron sulfur center and other possible models for the oxidase are discussed.

Methods

Cell culture. The cells used were the wild-type strain, RL 21a, and the poky strain NSX f^+a, both of which were the kind gift of Dr. C. Slayman and Dr. A. Lambowitz. Procedures for the maintenance of strains and the preparation of conidia have been described by Slayman and Tatum [15].

The cells were grown in aerated-liquid culture at 25°C in Vogels minimal medium, plus 2% sucrose, generally minus manganese [16]. Cells were harvested in late exponential phase, 15 h for wild type and 21 h for poky [17].

Preparation of mitochondria. The procedure used was the snail enzyme method of Smith [18] as modified by Lambowitz et al. [1]. Generally 10—25 g cells were harvested from 4 l growth medium and these yielded approx. 30 mg of mitochondrial protein.

EPR measurements. Samples for EPR measurements of mitochondria and whole cells in various states were frozen rapidly in liquid nitrogen and stored in liquid nitrogen until assayed. All spectra were obtained with a Varian E-4 or E-109 EPR spectrometer (Varian Associates). The temperature of the samples for EPR measurements were controlled with a variable temperature cryostat (Air Products Model LTD-3-110). Temperature was monitored with a carbon resistor placed in the helium flow directly below the sample and g-values were corrected by reference to a weak pitch standard. Quartz glass EPR sample tubes were calibrated with a standard copper sulphate-EDTA solution and values for signal heights were corrected correspondingly when relative quantitation was required.

Oxygen consumption. Oxygen consumption was measured with mitochondria or submitochondrial particles resuspended to an appropriate protein concentration in a medium containing 0.3 M mannitol/10 mM KCl/5 mM $MgCl_2$/10 mM potassium phosphate, pH 7.2. A Clark-type oxygen electrode, manufactured by Hansatech Limited, was used for the measurements.

For inhibitor studies, the mitochondria were always preincubated with inhibitor before substrate was added so that maximum inhibition was obtained.

Others. Standard chemicals used were of the highest grade available commercially. Protein was measured by the method of Lowry et al. [19] with crystalline bovine serum albumin as a standard.

Results

Characterization of mitochondria

The mitochondria isolated from wild-type *N. crassa* did not have unusual respiratory activities. They had a normal complement of cytochromes *a*, *b* and *c*, oxidized NADH and succinate at State 3 rates of around 275 and 170 nmol O_2 consumed/mg protein per min at 25°C, respectively, and were more than 98% inhibited by 1 mM KCN or 1 μg/ml antimycin A.

Mitochondria isolated from the poky strain of the organism were severely deficient in cytochromes aa_3 and *b*, had an elevated level of cytochrome *c*, and were more than 85% insensitive to either 1 mM KCN to 1 μg/ml antimycin A (added before substrate to ensure maximum inhibition). This KCN- and antimycin A-insensitive rate was largely inhibited by 1 mM salicylhydroxamic acid and hence was proceeding via the alternative oxidase. Rates of oxygen consumption of around 285 and 180 nmol/mg protein per min at 25°C with NADH and succinate as substrates, respectively, were routinely obtained.

These characteristics are similar to those reported previously by other workers [1] and confirmed that we had a controlled system with which to work.

The EPR-detectable components of whole poky cells

In order to gain some idea of the types of components present in poky cells, an oxygenated sample of the whole cells was frozen rapidly in liquid nitrogen. Fig. 1 illustrates the low temperature (8 K) spectrum of this sample. In the high

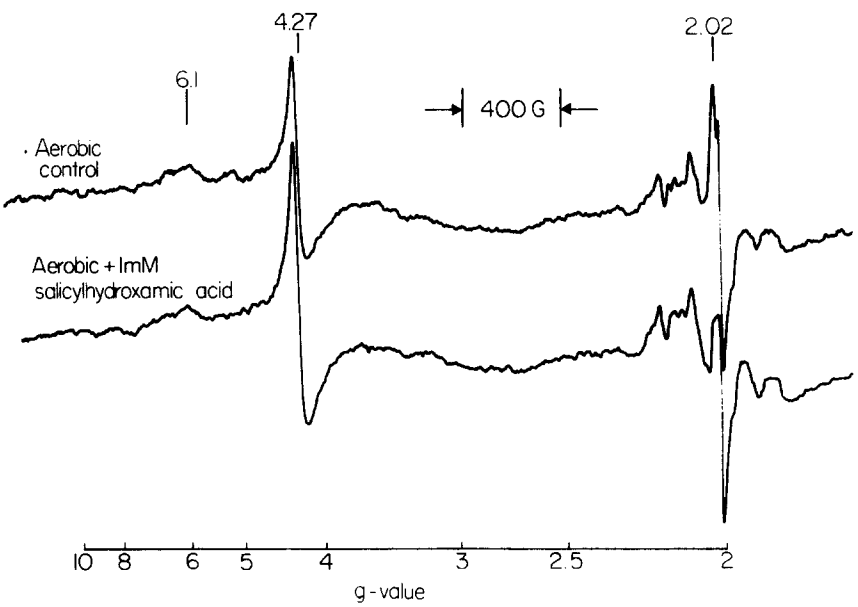

Fig. 1. EPR spectrum at 8 K of aerobic whole cells of poky *N. crassa*. Samples of poky *N. crassa* cells (approx. 250 mg wet weight of cells/ml) were gassed with pure oxygen for 15 s, rapidly transferred to quartz EPR tubes, and frozen with liquid nitrogen. Conditions of EPR measurement were: microwave power, 5 mW; microwave frequency, 9.17 GHz; modulation amplitude 12.5 G; modulation frequency, 100 KHz; gain, $2 \cdot 10^3$; temperature, 8 K.

field end and stretching over the $g = 2.3$ to $g = 1.98$ region is the multi-featured spectrum of manganese. It was found that if manganese were removed from the culture medium, this signal was absent from EPR samples, and no apparent harm or change was detectable in the cells. Hence, in cultures grown for mitochondrial preparation, manganese was omitted so that this interference was avoided.

Superimposed on the manganese signal was a sharp feature around $g = 2.02$. This signal was observable only at low (<20 K) temperatures and was partially overlapped by a large free radical signal at $g = 2.00$. The $g = 2.02$ signal is ascribed to the mitochondrial HiPIP-type * Center S-3 of succinate dehydrogenase [20,21].

Further features could be observed at lower field positions (Fig. 1). A prominent signal peaking at $g = 4.3$, attributable to high spin iron in an environment of low symmetry [22], was always present. Signals of this type can be seen in most biological systems and are generally considered to be non-physiological. Certainly, the signal could not be rapidly reduced to a significant extent by succinate or sodium dithionite, an observation which indicated a lack of interaction with the respiratory chain. In the $g = 6$ region of the spectrum it was possible to discern a variety of features. Their exact nature was somewhat variable between samples and they were attributed to oxidized high spin and mixed spin heme species (cf. ref. 23). It is already known that a small perturbation of the environment around these species can have dramatic effects on the exact positions and intensities of their signals [24,25].

Of interest was the finding that addition of salicylhydroxamic acid to the aerobic poky cells caused a loss of the $g = 2.02$ (HiPIP-type Center S-3) signal (Fig. 1), presumably because of reduction of the center to its diamagnetic state. This demonstrates that the Center S-3 is involved physiologically in electron transport from substrate to the alternative oxidase. Apart from a slight increase in the $g = 4.3$ iron signal, no other large changes in EPR-detectable components on addition of salicylhydroxamic acid were observed, whether the cells were in the oxidized or reduced states.

The EPR-detectable components of isolated aerobic poky mitochondria

Fig. 2 illustrates the major EPR signals observable in an aerobic sample of mitochondria isolated from poky cells. The cells were cultured in the absence of manganese since it was found that, as in the case of whole cells, a large contaminating manganese signal would otherwise be present in the mitochondrial samples.

At the low field end of the spectrum, signals attributable to high spin heme components similar to those observed in whole cells were present, although their ratio with respect to Center S-3 was much less than in whole cells (cf. Figs. 1 and 2). The exact magnitudes of the species were somewhat variable between samples but basically consisted of a rather axial component with

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* HiPIP is a generally accepted abbreviation for a class of iron sulfur proteins which are paramagnetic in their oxidized form. The abbreviation is derived from the first of this type to be detected, which was termed a high potential iron sulfur protein [45]. The use of the nomenclature in this manuscript does not imply any redox information.

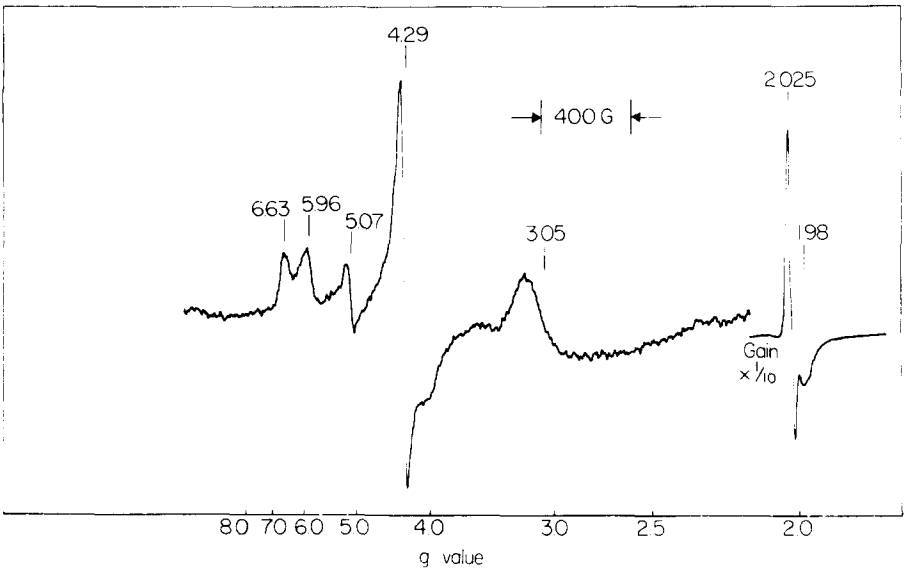

Fig. 2. EPR spectrum at 8 K of aerobic mitochondria isolated from poky *N. crassa*. A sample of poky *N. crassa* mitochondria was diluted to ≈30 mg protein/ml with 0.3 M mannitol/10 mM KCl/5 mM $MgCl_2$/ 10 mM potassium phosphate, pH 7.2. The sample was then gassed with pure oxygen for 15 s, transferred to a quartz EPR tube and frozen rapidly in liquid nitrogen. Conditions of EPR measurement were: microwave power, 5 mW; microwave frequency, 9.182 GHz; modulation amplitude, 12.5 G; modulation frequency, 100 KHz; gain, $1.25 \cdot 10^4$ ($g = 8$ to $g = 2.2$) or $1.25 \cdot 10^3$ ($g = 2.1$ and below); temperature, 8 K.

g-values around 5.96 and a second rhombically distorted signal with *g*-values at around 6.6 and 5.07.

The $g = 4.3$ signal was also present in these samples, although its ratio with respect to Center S-3 was about an order of magnitude less than in the whole cells (cf. Figs. 1 and 2). The signal appeared to be somewhat heterogenous, being composed of a sharp signal and an overlapping broader component.

Also present was a fairly broad signal which peaked at around $g = 3.15$—3.2. Similar signals have been detected in a number of systems and have been attributed to low spin cytochrome species [26—28]. In the case of poky mitochondria, the only cytochrome present in significant amounts was cytochrome *c* and hence we tentatively assign this low spin heme signal to cytochrome *c*.

By far the largest signal, however, was that peaking around $g = 2.02$ and caused mainly by HiPIP-type Center S-3 of succinate dehydrogenase at this temperature (8 K). No signals attributable to interacting ubisemiquinone species (cf. refs. 29, 30, 12) could be observed in any samples. The spectrum, however, was not a pure Center S-3 spectrum. The most prominent anomaly was an extremely broad underlying signal which had a peak in the same region as the Center S-3 peak and a trough at around $g = 1.97$—1.98. This novel distortion warranted further investigation.

In Figs. 3 and 4, the $g = 2$ region of the spectrum is analyzed in more detail. In Fig. 3, a temperature profile of the HiPIP region of the spectrum is illustrated for both poky and wild-type mitochondria. The microwave power was

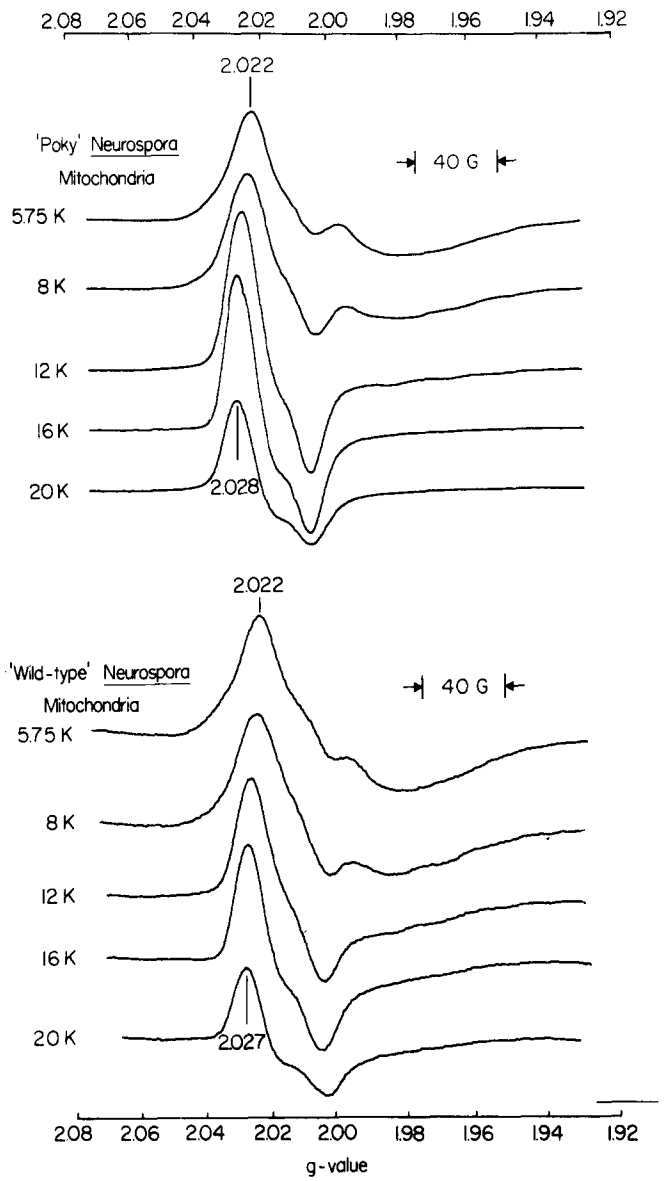

Fig. 3. Temperature profiles of HiPIP-type EPR spectra of aerobic poky and wild-type *N. crassa* mitochondria. Samples of mitochondria were diluted to 30 mg/ml (poky) or 56 mg/ml (wild-type) with 0.3 M mannitol/10 mM KCl/5 mM $MgCl_2$/10 mM potassium phosphate at pH 7.2. The samples were gassed with pure oxygen for 15 s, transferred to quartz EPR tubes, and frozen rapidly in liquid nitrogen. Conditions of EPR measurement were: microwave power, 5 mW; microwave frequency, 9.185 GHz; modulation amplitude, 12.5 G; modulation frequency, 100 KHz; gain, wild type $2 \cdot 10^3$, poky, $8 \cdot 10^2$.

fixed at 5 mW, and although some of the components were partially saturated at low temperatures, variation of temperature of measurement at this power served to distinguish the multiple components which comprised the spectra. At low temperatures (<10 K) the spectrum comprised mainly a peak around $g = 2.02$, together with an underlying broad trough extending over $g = 1.99$—

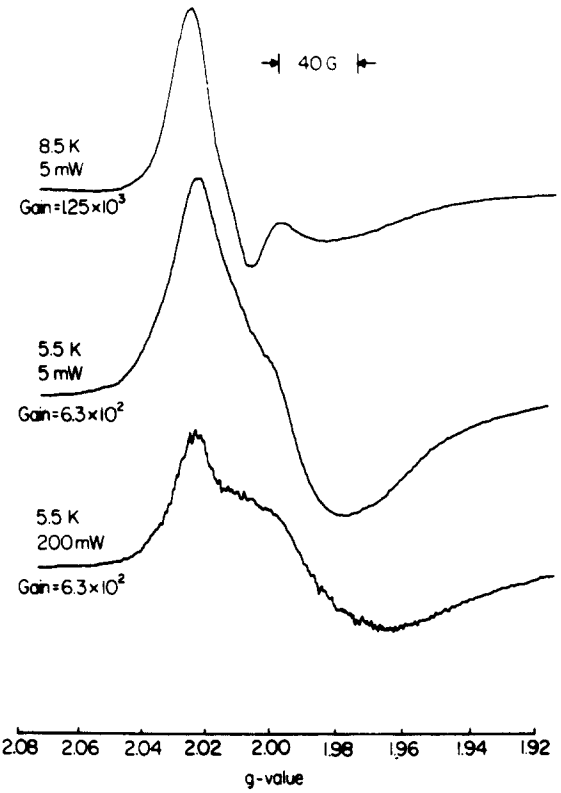

Fig. 4. Resolution of an underlying broad component in the HiPIP spectrum of poky *N. crassa* mitochondria. Mitochondria (30 mg protein/ml) were oxygenated for 15 s, transferred to a quartz EPR tube and frozen in liquid nitrogen. Both spectra were taken with: microwave frequency, 9.182 GHz; modulation amplitude, 12.5 G; modulation frequency, 100 KHz. Other conditions of measurement are given with the appropriate spectrum. The upper spectrum is comprised mainly of Center S-3, whereas this Center S-3 is mostly saturated in the lower spectrum and leaves the broad underlying component clearly distinguishable.

1.94. As the temperature was raised, this broad component rapidly diminished and was almost undetectable by 12 K. The peak remaining at this temperature was apparently rather narrower than at lower temperatures because of the absence of the broad component. As the temperature was raised further the peak position approached a value around $g = 2.03$. This signal rapidly diminished at temperatures above 20 K, leaving only a free radical $g = 2.00$ component by 30 K. No significant differences were noted between the poky and wild-type *N. crassa* mitochondria.

It is thought that three major components comprise these spectra, (a) a normal HiPIP-type Center S-3 [20,21] with $g_{max} = 2.02$ and which is maximal around 10 K at 5 mW power; (b) a Ruzicka-type HiPIP [31,32] with g_{max} closer to 2.03 and which is maximal at rather higher (approx. 15 K) temperatures; (c) a severely distorted Center S-3 species which has a broad peak in the g 2.04—2.00 region and a broad trough in the $g = 1.99$—1.94 region. This signal is very rapidly relaxing and was not fully saturated even at 5.8 K with 5 mW of micro-

wave power. By raising the power and lowering the temperature so that the undistorted Center S-3 was mainly saturated out, the broad underlying signal (itself partially saturated at 200 mW power and 5.5 K) could be clearly seen (Fig. 4). The signal was centered at $g = 1.99$ and had an approximate peak to trough width of 80 gauss.

It is thought that this distortion is caused by an interaction of the Center S-3 with a semiquinone species. Preliminary evidence for this notion has come from an experiment where the poky mitochondria were aged overnight at 4°C. This treatment did not affect the total activity of the cytochrome oxidase pathway with succinate as substrate but did presumably cause some degeneration of the mitochondrial structure. It was found that this treatment caused a large increase in the amount of 'normal' Center S-3 and a decrease in the amount of 'distorted' Center S-3. Coupled to this conversion was the loss of a free radical species which was observable at higher temperatures (Fig. 5). The free radical was centered around $g = 2.005$ and had a peak to trough width of approx. 10 gauss. It is proposed that this free radical signal is caused by a stable ubisemiquinone species and that the interaction is an analogous phenomenon to that which causes the semiquinone-semiquinone spin-spin interaction in mammalian and higher plant systems (cf. refs. 29, 30, 12). In the *N. crassa* case, however,

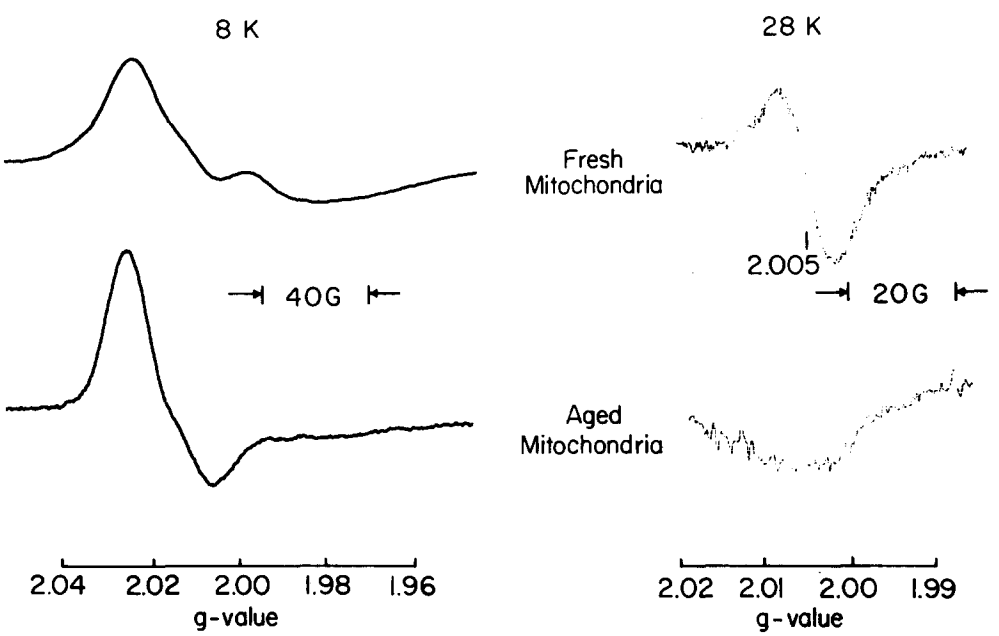

Fig. 5. The relation of the HiPIP spectrum to the presence of a free radical $g = 2.00$ component in fresh and aged poky *N. crassa* mitochondria. A sample of fresh mitochondria (approx. 8 mg/ml) was oxygenated for 15 s, transferred to a quartz EPR tube and frozen rapidly in liquid nitrogen (upper spectra). A second identical sample (same protein concentration), but which had been aged overnight at 4°C as a 24 mg/ml pellet, was frozen under the same conditions (lower spectra). HiPIP (8 K) spectral conditions were microwave power, 5 mW; microwave frequency, 9.108 GHz; modulation amplitude, 12.5 G; modulation frequency, 100 KHz; gain, $5 \cdot 10^3$. Radical (28 K) spectral conditions were: microwave power, 1 mW; microwave frequency, 9.108 GHz; modulation amplitude, 5 G; modulation frequency, 100 KHz; gain, $1.6 \cdot 10^4$.

the interaction is somewhat different in that it is the Center S-3 itself which is the distorted species. Whether this interaction is a magnetic or a conformational phenomenon is unclear at present. It should be noted that the interaction and distortion of Center S-3 also occurred in the wild-type mitochondria (see Fig. 3) and was not a unique property of the poky system. The presence of the interaction was dependent on the quality of the mitochondria and generally any treatment which damaged the membranes also caused a reversion of the distorted Center S-3 to its more normal state. A more detailed treatment of this interaction will be presented elsewhere.

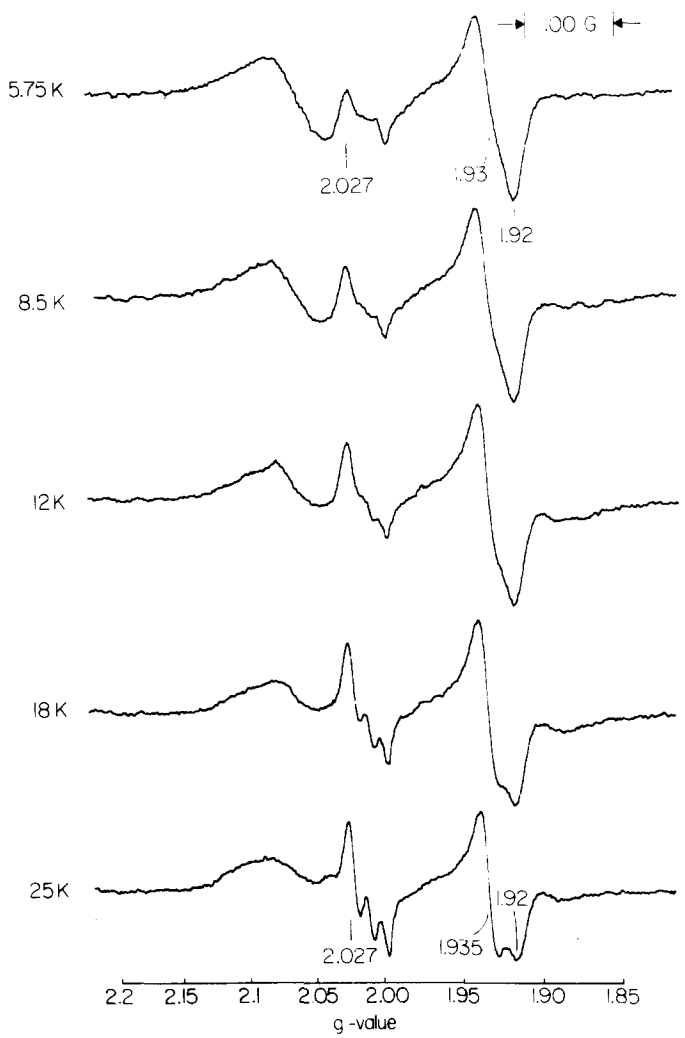

Fig. 6. EPR spectra of dithionite-reduced poky *N. crassa* mitochondria. A sample of mitochondria (43 mg/ml) in 0.3 M mannitol/10 mM KCl/5 mM $MgCl_2$/10 mM potassium phosphate, pH 7.2, was reduced with a little solid sodium dithionite for 1 min at room temperature, transferred to a quartz EPR tube, and frozen rapidly in liquid nitrogen. Conditions of EPR measurement were: microwave power, 5 mW; microwave frequency, 9.108 GHz; modulation amplitude, 12.5 G; modulation frequency, 100 KHz; gain, $5 \cdot 10^3$.

The EPR-detectable components of isolated reduced poky mitochondria

Fig. 6 illustrates the paramagnetic components which were observable in the reduced state. A sample of poky mitochondria were reduced with a few crystals of solid sodium dithionite and the sample was left at room temperature for 1 min before rapid freezing in liquid nitrogen. A temperature profile of this sample revealed the presence of only two major ferredoxin-type centers. The first of these, observable at low temperatures, had features at $g = 2.03$ and 1.93. As the temperature was raised above 10 K, the signal shape changed in the $g = 1.93$ region to produce a much more rhombic signal with $g_y = 1.935$ and $g_x = 1.92$. By 25 K, this rhombic component was the dominant signal. When succinate was used as reductant in place of sodium dithionite, only the more slowly relaxing component (i.e., rhombic signal at 25 K) was detected in significant amounts. It is proposed that these features are caused by centers equivalent to Centers S-1 ($g = 2.03$, 1.935, 1.92, maximal around 25 K) and S-2 ($g = 2.03$, 1.93, maximal below 10 K) of succinate dehydrogenase (cf. refs. 21 and 33). No other ferredoxin-type centers could be detected in significant amounts. It is probable that the Center S-2 is actually rhombic, but has been distorted by saturation at these temperatures and powers (cf. ref. 33).

The effects of salicylhydroxamic acid on the EPR signals of poky mitochondria

Salicylhydroxamic acid is a well-known inhibitor of the alternative oxidase [9]. Many workers assume that its mode of action is by metal chelation although we [25] have recently proposed an alternative hypothesis of a competition of the inhibitor with the reducing substrate (in this case ubiquinol, refs. 35, 36) at its binding site. The effects of this inhibitor were tested on the EPR signals that we were able to detect.

As expected from our previous results and predictions on the mechanism of action of hydroxamic acids on redox systems [25,34], no discernible effect of 1 mM salicylhydroxamic acid could be found on either of the two major ferredoxin-type centers (Centers S-1 and S-2 of Fig. 6) that are present in the poky mitochondria. This indicates that a ferredoxin-type iron sulfur center is not directly involved in the terminal alternative oxidation step and that the hydroxamic acid is not acting by metal chelation of such a component.

The effects of 1 mM salicylhydroxamic acid were also tested on the components which could be detected in the aerobic state. In a temperature profile of the HiPIP-type centers (cf. Fig. 3), no effect of the inhibitor on either the position, shape, or relaxation behavior of either of these centers could be discerned. This is consistent with our previous studies on the analogous compoments in higher plant systems [34]. The loss of the HiPIP signal observed in whole *N. crassa* cells on addition of a hydroxamic acid is presumably because of reduction of the Center S-3 by the endogenous substrates of the intact cells (see Fig. 1). These substrates are not present in the mitochondrial fraction, however, and hence the Center S-3 remains oxidised in the presence of the alternative oxidase inhibitor. Some small change in shape of the total spectrum in this region could be observed, however. This small change, which was rather difficult to qualitate, was attributed to a hydroxamic acid effect on a part of the distorted Center S-3 signal, causing it to revert to the more normal Center S-3 signal shape. This was coupled with a small decrease in the free radical $g =$

2.005 signal which was observable at higher temperatures (cf. Fig. 5). The effect is analogous to the rather non-specific effect of salicylhydroxamic acid on the semiquinone spin-spin interaction in plant mitochondria [12] and may represent a competition of the hydroxamate with ubiquinone for its binding site [25], hence destabilizing the semiquinone form.

A further small effect of salicylhydroxamic acid could be seen on the $g = 4.3$ iron signal, generally causing an approx. 15—20% increase of the signal. This may be caused by the rather unspecific ferric iron chelating ability of the hydroxamic acid [37].

Some decrease in one of the high spin heme signals ($g = 5.96$, axial signal) was noted on addition of hydroxamic acid (Fig. 7). This may have been caused by either environment change or reduction of the ferric moiety. We have already reported the distortion of the high spin heme of horseradish peroxidase when salicylhydroxamic acid binds to the protein [25].

No other significant effects of hydroxamic acid addition could be found.

The effect of succinate reduction on redox components

It is well-known that succinate is able to efficiently support maximal flux through the alternative oxidase pathway under appropriate conditions. Use was made of this to determine which of the EPR-detectable components could possibly be involved in the alternative pathway, since only those which are

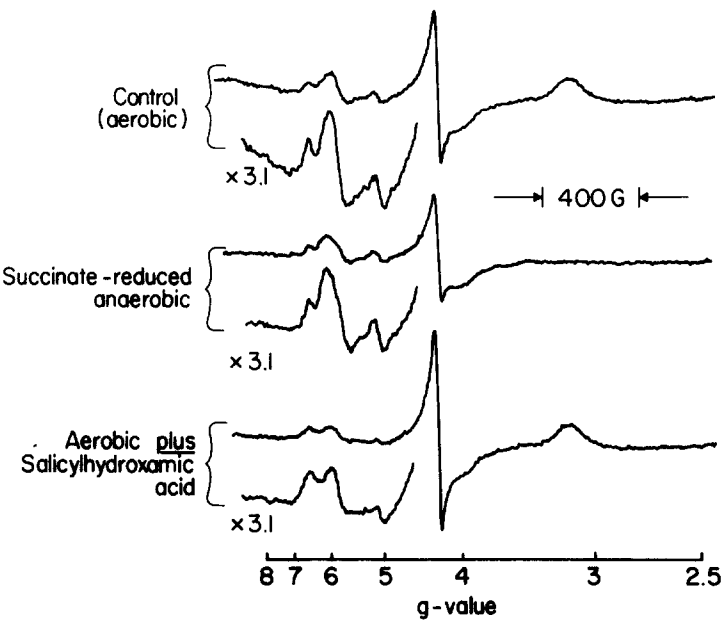

Fig. 7. The effects of succinate reduction and salicylhydroxamic acid on the low field EPR signals of poky *N. crassa* mitochondria. Samples of poky *N. crassa* mitochondria (approx. 15 mg/ml) were frozen in liquid nitrogen under the following conditions: top trace, aerobic control, after gassing for 15 s with oxygen; middle trace, after respiration to anaerobiosis with 10 mM succinate as substrate; bottom trace, after gassing for 15 s with oxygen in the presence of 1 mM salicylhydroxamic acid. The medium used was 0.3 M mannitol/10 mM KCl/5 mM $MgCl_2$/10 mM potassium phosphate, pH 7.2. Conditions of EPR measurement were: microwave power, 5 mW; microwave frequency, 9.108 GHz; modulation amplitude, 12.5 G; modulation frequency, 100 KHz; temperature, 5.5 K; gain, $2 \cdot 10^3$.

efficiently reducible by succinate need be considered. Hence an experiment was performed in which the EPR spectra of a sample which had been allowed to respire to anaerobiosis with succinate as substrate was compared to an aerobic (no substrate) control sample. The results are illustrated in Fig. 7.

The components which were succinate-reducible were, (i) all of the HiPIP and distorted HiPIP signals (not shown); (ii) the ferredoxin-type center which was maximal above 20 K at 5 mW power, presumably Center S-1 (cf. refs. 21 and 33); (iii) the low spin heme (cytochrome *c*) signal which peaked at $g = 3.15$; (iv) a fraction (20—30%) of the $g = 4.3$ high spin iron complement.

No other components were reduced to a significant extent and these succinate-non-reducible moieties could therefore be ruled out as possible components of the alternative pathway from succinate to molecular oxygen. It should be noted, of course, that succinate-reducibility of a component does not prove that the component is involved in the alternative pathway.

Redox properties of the paramagnetic centers

A sample of mitochondria was diluted into 50 mM Tris buffer at pH 7.5 and an anaerobic redox titration in the presence of redox mediators [38] was performed in the potential range +200 to —400 mV. Mediators used were: methyl viologen, benzyl viologen, phenosafranine, anthraquinone-2-6-disulfonate, 2-hydroxy-1-4-naphthoquinone, indigotetrasulfonate, pyocyanine, duroquinone, *N*-ethyl phenazonium ethosulfate, *N*-methyl phenazonium methosulfate, and diaminodurol (all at 10—25 μM concentration). 1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was added as an uncoupler. Samples for EPR measurement were taken anaerobically at appropriately poised potentials and were frozen rapidly in liquid nitrogen. Potential was raised or lowered by addition of aliquots of potassium ferricyanide or sodium dithionite respectively. The redox state of each component was determined by measurement of its EPR spectrum under appropriate conditions. The accumulated results of these titrations are illustrated in Fig. 8.

Fig. 8A illustrates the redox dependence of the HiPIP-type Center S-3 signal (measured as the $g = 2.02$ peak height at 6 K). As is typical of these Centers, the signal exists only in the oxidized state. The curve drawn is that theoretically calculated for an $n = 1$ redox component and from the best fit of this curve, a midpoint potential of $+40 \pm 20$ mV at pH 7.5 and 25°C was found.

In Fig. 8B, the redox dependencies of the ferredoxin-type iron sulfur center signals are illustrated. These were measured as a peak to trough signal size around $g = 1.93$. Three temperatures of measurement were used so that multiple centers might be distinguished. The results of these titrations however, confirmed our original analysis that only two major ferredoxin-type centers, Centers S-1 and S-2, were present. Hence, at low temperature (6 K) virtually the only center observable was the low potential, rapidly relaxing Center S-2 (Center S-1 was almost fully saturated under these conditions). As the temperature was raised, however, a larger contribution to the total ferredoxin signal was provided by the higher potential, more slowly relaxing Center S-1, until at 22 K this was the dominant center. This difference was also reflected in the shape of the signal of a fully reduced sample, which changed from a rather rhombic form at high (22 K) temperature (i.e., mainly Center S-1) to an

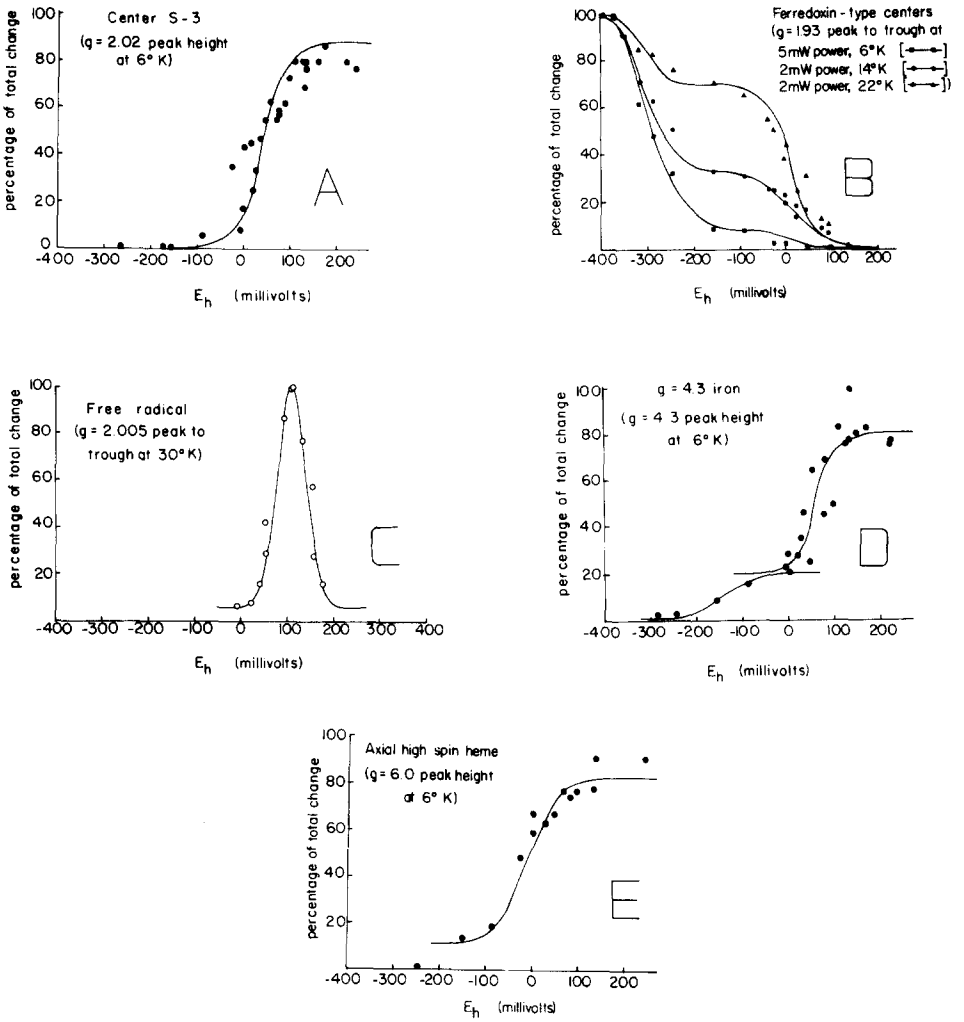

Fig. 8. Redox titrations of the paramagnetic centers associated with poky *N. crassa* mitochondria. Mitochondria were diluted, generally to approx. 10 mg/ml, in an anaerobic medium of 50 mM Tris buffer at pH 7.5 and 25°C together with appropriate mediators (see text). The whole was made anaerobic with a flow of argon gas and potential was monitored with a platinum electrode against a calomel standard. Sodium dithionite or potassium ferricyanide were used to change potential. Samples were transferred to quartz EPR tubes anaerobically and frozen rapidly in liquid nitrogen. The results were the accumulated data of at least three experiments of both oxidative and reductive titrations for each component and the curves are theoretically calculated curves for $n = 1$ redox components.

apparently more axial shape at 6 K (i.e., mainly Center S-2). The midpoint potentials of the two centers measured from these experiments were −310 ± 20 and +10 ± 20 mV at pH 7.5 and 25°C for Centers S-2 and S-1, respectively.

In Fig. 8C, the redox dependence of the free radical signal is illustrated (measured as the peak to trough size of the signal around $g = 2.005$ at 30 K). The component displays two midpoint potentials, characteristic of an $n = 2$ redox center which has diamagnetic oxidized and reduced states, but which has

a stable, paramagnetic radical form. The component is assigned to the semiquinone form of ubiquinone on the basis of signal position, shape and redox properties. The two apparent $n = 1$ half potentials at pH 7.5 were measured as +75 mV (quinol/semiquinone couple) and +140 mV (semiquinone/quinone couple). (The half potentials that we observe are only apparent since we never observe an amount of semiquinone equal to the total quinone present, because of the overlapping of the two potential curves). We have previously observed a similar component in higher plant systems [14,34].

Fig. 8D illustrates the redox behavior of the $g = 4.3$ iron signal (measured as the $g = 4.3$ peak height at 6 K). The signal is apparently heterogeneous, being composed of at least two components, a major one with a midpoint potential at pH 7.5 of +60 mV and a second minor component with a midpoint potential at pH 7.5 of around —150 mV. The signal was found to equilibrate only very sluggishly with our mediator system and so a long time was allowed for equilibrium to be reached. From the changes in signal shape, it was apparent that the sharper component of the $g = 4.3$ spectrum was the +60 mV species and the broader $g = 4.3$ component was the —150 mV species.

We were unable to accurately measure the redox behavior of the small high spin heme signals around $g = 6$. It appeared, however, that the more axial component was rather heterogeneous, but had a major portion with a midpoint potential around -10 ± 20 mV at pH 7.5 and 25°C (Fig. 8E).

We were unable to detect the $g = 3.15$, low spin heme, signal, even at the highest potential at which we began the titration and under conditions optimal for observation of the signal (low powers around 20 K). We therefore assume that the midpoint potential of the center is greater than +200 mV at pH 7.5, a result which is consistent with its identification as cytochrome *c*. It was experimentally difficult to maintain stable potentials above +200 mV because of the presence of endogenous reductants in the mitochondria which caused a rapid drift downwards of the potential of the system. Hence, an accurate determination of the midpoint potential of the $g = 3.15$ peak was not feasible.

Discussion

The poky *N. crassa* mitochondria offer an excellent controlled system for the study of alternative respiratory oxidations since one is always able to refer back to the normal wild-type system in cases of ambiguity. This study represents an initial analysis of the paramagnetic centers of the poky system and provides a framework on which to base further investigations. Table I summarizes the components that we were able to identify in this analysis and lists some of their properties.

It is apparent that the major routes of electron transport in the poky mitochondria are those from either succinate or external NADH to molecular oxygen via the alternative oxidase. This is strongly reflected in the types of iron sulfur centers present, the large excess of Centers S-1, S-2 and S-3 of succinate dehydrogenase and the very low levels of Site I iron sulfur centers and the Rieske Center (see Table II). Of interest is the fact that no particular iron sulfur center could be attributed to the pathway of external NADH oxidation, a result which suggests that the external NADH dehydrogenase may

TABLE I

A SUMMARY OF THE PARAMAGNETIC CENTERS ASSOCIATED WITH POKY *N. CRASSA* MITOCHONDRIA

Component	Features	Useful temperature of observation (K)	$E_{m\ 7.5}$ (mV)
Center S-1	$g_x = 1.92, g_y = 1.935, g_z = 2.03$	25	+10 ± 20
Center S-2	$g = 1.93, 2.03$	<10	−310 ± 20
Center S-3	$g_{max} \approx 2.02$	10	+40 ± 20
Ruzicka Center	$g_{max} \approx 2.03$	20	n.d.
$g = 2.00$ free radical	$g = 2.005$	30	Apparent E_{m1} = +75 ± 10 ($QH \cdot / QH_2$) Apparent E_{m2} = +140 ± 10 ($Q/QH \cdot$)
$g = 4.3$ iron	centered around $g = 4.3$	25	(i) +60 ± 20 (ii) −150 ± 30
Axial high spin heme	centered around $g \approx 5.96$	<10	−10 ± 20
Rhombic high or mixed spin heme	$g_x \approx 6.5, g_y \approx 5.1$	<10	n.d.
Low spin heme	$g_z = 3.15$	25	>200

n.d., not determined.

TABLE II

A COMPARISON OF THE MAJOR REDOX COMPONENTS ASSOCIATED WITH WILD-TYPE AND POKY *N. CRASSA* MITOCHONDRIA

The positive symbol denotes that the component is present in significant amounts, whereas the negative symbol denotes that the component is in low amounts or absent.

Component	Wild-type *N. crassa*	Poky *N. crassa*
Cytochromes *		
aa_3	+	−
b-type	+	−
c-type	+	+
Ferredoxin-type centers **		
Site 1 (N-1 to N-4)	+	−
Succinate dehydrogenase (S-1, S-2)	+	+
Rieske Center	+	−
'Center 5'	+	−
HiPIP-type centers ***		
Center S-3	+	+
'Ruzicka'-type Center	+	+
Others **		
High spin hemes	+	+
$g = 4.3$ iron	+	+
$g = 2.00$ semiquinone	+	+

* Data from ref. 1.
** Data from ref. 3 and this work.
*** Data from this work.

donate electrons directly to ubiquinone. This result is in contrast to the recent suggestion of Cammack and Palmer [39] that a novel iron sulfur center may be involved in the higher plant external NADH oxidase pathway.

The distortion of the Center S-3 spectrum in freshly prepared poky and wild-type mitochondria is a novel finding. Preliminary data suggest to us that the distortion is caused by an interaction with a moiety containing a semiquinone species, although whether the interaction is paramagnetic or conformational is still a moot point. In any case, the study of this interaction will provide information on the linkage of Center S-3 to the rest of the respiratory chain and may provide insight into the semiquinone spin-spin interaction which has previously been observed in other systems [29,30,12]. It is still unclear whether the spins of the distorted signal arise solely from the Center S-3 or whether the signal is more analogous to this semiquinone spin-spin interaction. Further investigations are necessary to clarify this point.

The rather high concentration of cytochrome *c* in the poky mitochondria, together with the very small levels of cytochromes *b* and aa_3, adds another novel feature to the EPR spectrum, that of a fairly clean low spin heme signal peaking around $g = 3.15$. In other systems the signal is much more difficult to detect since it is in lower concentrations and is accompanied by the g_z and g_y components of the other cytochromes (cf. ref. 28). This feature of poky mitochondria may provide a means of studying the in situ location and orientation of cytochrome *c*.

The $g = 4.3$ iron signal is considered to be of doubtful physiological significance. Such signals are routinely observed in many biological specimens and may represent non-specifically bound iron. Even in purified systems where the iron is stoichiometric with protein, such as aconitase [40], the $g = 4.3$ iron has doubtful physiological meaning. In our systems the partial loss of signal with succinate probably represents a chemical reduction of the iron and the increase in signal with salicylhydroxamic acid probably is a manifestation of the ferric iron chelating ability of the hydroxamic acid. Some degree of heterogeneity of this iron does exist, as is seen in the shape and the redox properties of the signal.

The high spin heme signals associated with the mitochondrial system are difficult to identify. Since they were not reduced by succinate, it is likely that they do not represent mitochondrial cytochromes, but instead may belong to other redox enzymes, such as peroxidases and catalases in the case of the low potential component(s), or perhaps to nascent or denatured cytochromes. The possibility exists that they are bound to a contaminating factor in our mitochondrial preparation.

The relation of the signals to the alternative oxidase

No paramagnetic centers have been identified in the poky *N. crassa* mitochondria which we are not able to ascribe to previously known types of components. Although this is a rather negative finding in terms of the nature of the alternative oxidase, it allows us at least to narrow down the possibilities as far as its structure is concerned. For example, it is now clear that it is not a ferredoxin-type or HiPIP-type iron sulfur center, although this hypothesis has tended to gain a general acceptance among workers in the field. In fact, it

seems rather likely that the oxidase itself, both in its native oxidized and reduced states, is diamagnetic. This result, taken together with its optical invisibility and with the finding that it donates at least two, perhaps four, electrons to molecular oxygen [41,42], allows us to speculate to some extent upon the limited number of possible structures, which it migh have, based upon structures of the various classes of redox enzymes known to date. At present, we are considering and carrying out investigations which might allow us to identify one of the following types of structure as the terminal oxygen-consuming system: (a) an autoxidisable quinol/quinone system, such as we have previously considered in connection with the analogous higher plant system [14]. Identification and characterization of the redox chemistry of the quinones present in the poky system will clarify this hypothesis; (b) a copper dimer system, the spin coupling between which makes the system EPR silent, in a manner similar to some other known copper-containing oxidases (cf. refs. 43 and 44); (c) a non-heme and non-iron sulfur, iron-containing system, in which the iron is held in a reduced or unusual spin coupled state so that it is EPR-silent.

Acknowledgments

The authors are indebted to Drs. C.W. Slayman and A. Lambowitz for the supply of wild type and mutant strains of *N. crassa*, to Ms. N.K. Wiegand for expert technical assistance and to Ms. M. Mosley for careful preparation of the manuscript. The work was supported by grants from the National Science Foundation and the Herman Frasch Foundation.

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