A SPIN 3/2 FERROUS–NITRIC OXIDE DERIVATIVE OF AN IRON-CONTAINING MOIETY ASSOCIATED WITH *NEUROSPORA CRASSA* AND HIGHER PLANT MITOCHONDRIA

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1. Introduction

We describe the detection of an iron-containing system associated with mitochondria isolated from *Neurospora crassa* and higher plant mitochondria by the technique of electron paramagnetic resonance. The component has been previously undetected since it is EPR-silent in its native state. Treatment with nitric oxide, however, produced a ferrous–NO derivative and gave a spin 3/2 iron signal with features at g_y 4.11, g_x 3.95 and g_z 2.00. The component was present in such large excess over the ferredoxin-type centers of the electron transport pathway that we suggest that the component responsible may be an iron storage or iron-transporting system associated with the mitochondria.

2. Methods

The *N. crassa* cells used were the wild-type strain RL 21a. Procedures for the maintainance of strains and the preparation of conidia have been described [1]. The cells were grown in aerated-liquid culture at 25°C in Vogels minimal medium, plus 2% sucrose [2], but minus manganese.

2.1. *Preparation of mitochondria*

Mitochondrial preparative procedures for *N. crassa* [3], higher plants [4] and beef heart [5] have been described. The mitochondria were diluted to an appropriate protein concentration into a medium containing 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl and 10 mM potassium phosphate, at pH 7.2, for assay.

2.2. *EPR samples and measurements*

Samples of mitochondria in various states were placed in quartz EPR tubes, frozen rapidly in liquid nitrogen, and stored at 77 K until assayed. All spectra were obtained with a Varian E-4 or E-109 EPR spectrometer (Varian Associates). The temperatures of the samples for EPR measurement 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.

The nitric oxide derivatives were prepared by generation of nitric oxide in situ, by addition of a few crystals of sodium nitrite and sodium dithionite. The nitrite was reduced to nitric oxide by the reductant.

2.3. *Protein and reagents*

Protein was assayed by the Lowry method [6]. All reagents were of the highest grade commercially available.

Abbreviations: EPR, electron paramagnetic resonance; NO, nitric oxide; EDTA, ethylenediamine tetraacetic acid

3. Results

The components seen in the oxidized and reduced spectra of *N. crassa* mitochondria have been described [7,8], and consisted mainly of high spin hemes (around *g* 6.0), *g* 4.3 iron and HiPIP-type center S-3 (*g* 2.02) in the oxidized state, and a number of ferredoxin-type centers in the reduced state. The most prominent feature of the nitric oxide-derivative spectrum, however, was that of a large signal with features at *g* 4.11, *g* 3.95 and *g* 2.00 (fig.1). These features were by far the largest of the spectrum. The component was very rapidly relaxing, and only began to saturate above 10 mW microwave power at 5.5 K. No nuclear hyperfine splitting of the spectrum could be observed (compare, e.g., the $\Delta m_s = 2$ transition of the copper dimer–NO complex of tyrosinases [9,10]). We were unable to attribute any signal to the component in either the dithionite-reduced or ferricyanide-oxidized condition in the absence of added nitric oxide, since none were large enough. However, since the '*g* 4.3' signal is not due to a ground state, the species associated with it may be present a larger quantity than is obvious from the signal. Also seen in the spectrum illustrated in fig.1 is some residual low potential low symmetry ferric iron signal around *g* 4.3 [8] and some ferredoxin-type iron–sulfur center signals around *g* 2 [7].

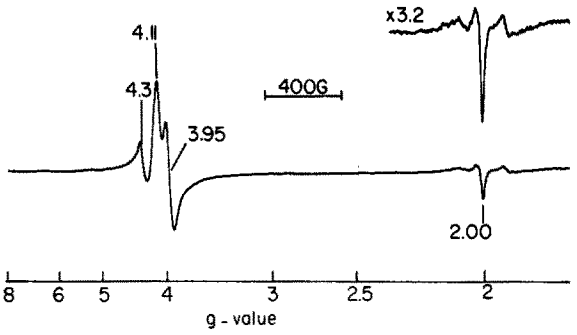

Fig.1. The EPR spectrum at 8.5 K of wild-type *N. crassa* mitochondria treated with nitric oxide. A sample of *N. crassa* mitochondria was diluted to ~10 mg protein/ml and transferred to a quartz EPR tube. A few crystals of solid sodium dithionite and sodium nitrite were mixed in, and after incubation for 30 s at 25°C, the whole was rapidly frozen in liquid nitrogen. Conditions of EPR measurement were: modulation frequency, 100 kHz; modulation amplitude, 12.5 G; microwave frequency, 9.10 GHz; microwave power, 10 mW; gain, 1.25×10^3; temp., 8.5 K.

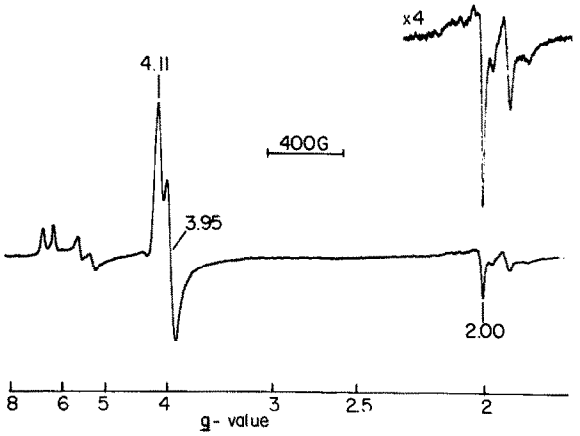

Fig.2. The EPR spectrum at 8.5 K of potato tuber mitochondria treated with nitric oxide. A sample of potato tuber mitochondria was diluted to ~15 mg protein/ml and treated with nitric oxide as described in fig.1. Conditions of EPR measurement were: modulation frequency, 100 kHz; modulation amplitude, 12.5 G; microwave frequency, 9.10 GHz; microwave power, 10 mW; gain, 10^3; temp. 8.5 K.

Figure 2 illustrates a similar nitric oxide derivative experiment performed with potato tuber mitochondria. It can be seen that an exactly analogous dominating component was present, again with *g*-values at 4.11, 3.95 and 2.00, together with some residual low potential high spin heme signals around *g* 6 and some ferredoxin-type centers around *g* 2. When a similar experiment was performed with intact beef heart mitochondria, no such dominant spin 3/2 component could be found.

A further experiment was performed with a model system – that of a ferric EDTA solution (fig.3). This was made by addition of 5 mM $FeCl_3$ to 50 mM EDTA at pH 7.0. The EPR spectrum of this compound (bottom trace, fig.3) is a typical *g* 4.3 low symmetry high spin iron. When nitric oxide was added, however (as solid sodium nitrite plus sodium dithionite), an EPR signal almost identical to that seen in the mitochondria was produced (top trace, fig.3) with its three *g*-values at 4.11, 3.95 and 2.00.

In order to determine the submitochondrial location of the component to some extent, a sample of *N. crassa* mitochondria was resuspended in distilled

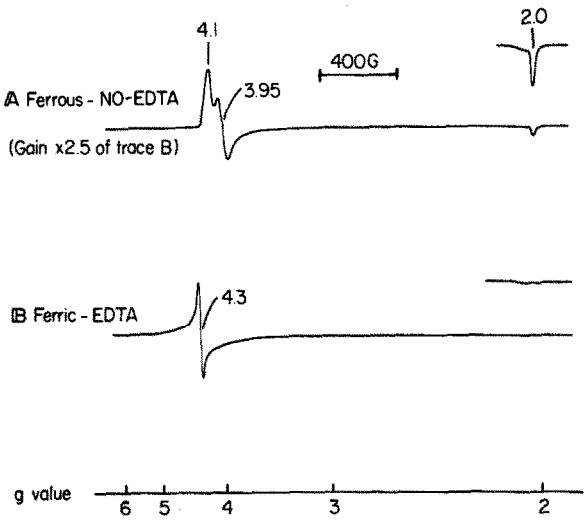

Fig.3. EPR spectra of iron-EDTA and its nitric oxide derivative. A solution of 5 mM ferric chloride in 50 mM EDTA at pH 7.0 was prepared. An aliquot of this was transferred to a quartz EPR tube and frozen rapidly in liquid nitrogen (trace B). A second aliquot was added to a matched tube and a few crystals of solid sodium dithionite and sodium nitrite were added. After 30 s incubation at 25°C, the sample was similarly frozen in liquid nitrogen (trace A). Conditions of EPR measurement were: modulation frequency, 100 kHz; modulation amplitude, 20 G; microwave frequency, 9.09 GHz; microwave power, 10 mW; gain, 125 (trace A) or 50 (trace B); temp., 7.5 K.

water to a final osmolarity of 35 mOsm and then French pressure cell-treated at 2000 p.s.i. so that the membranes ruptured to produce submitochondrial particles. During this treatment, the intramembranous soluble components were released into the supernatant. It was found that all of the component giving rise to the signal remained in the supernatant after centrifugation of the submitochondrial particles into a pellet. This indicated that the component was intramembranous and soluble, possibly in the mitochondrial matrix, although we cannot at present rule out a possible location in a contaminating organelle since the purity of *N. crassa* mitochondrial preparations have not been investigated in any detail. Crude plant mitochondrial preparations, for example, are known to have several other particles contaminating them [11].

4. Discussion

The component described represents a new class of metallocomplexes of biological origin which is detectable by EPR. The signal is attributed to a spin 3/2 iron-containing system, on the basis of the g position around g 4 and g 2.0. Of interest is the lack of nuclear hyperfine structure associated with the peaks. The close correspondence of its EPR spectra with that of the model ferrous–EDTA–NO complex is obvious. It is possible that the component is not detectable in the absence of nitric oxide, even in the presence of potassium ferricyanide, because it remains in the reduced state. Ferricyanide may not be able to oxidize it efficiently because of redox or accessibility factors. The component would presumably give a g 4.3 signal (cf. ferric–EDTA, fig.3), if it could be oxidized.

A similar signal formed during the catalytic cycle of nitrogenase has been noted ([12] cf. [13]). They attributed the signal to the g_x, g_y and g_z components of a spin 3/2 iron-containing system which was subjected to both axial and zero field splittings, and rationalized the unusual spin state of the iron as being caused by an exchange-coupled multiple iron system. Our results suggest that there may be several alternatives, either a single iron atom with a strong electron-accepting ligand or a multi-iron cluster involved in intermediate binding, in analogy to the Fe^{2+}–NO system that we observe, in addition to that pointed out in [12]. The iron might then remain reduced during the catalytic cycle and heme may account for the inactivation of nitrogenase on exposure to oxygen [14,15].

The amount of iron present in the component was at least an order of magnitude greater than that present in the total iron–sulfur centers of the respiratory chain, in both the potato and *N. crassa* systems. This leads us to consider that the component may be part of an iron-transporting system or an iron-storage system of the mitochondria.

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