EPR STUDIES OF ^{15}NO–FERROCYTOCHROME a_3 IN CYTOCHROME c OXIDASE

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1. Introduction

Cytochrome c oxidase is the terminal enzyme in the mitochondrial respiratory chain and, as such, catalyzes the 4 e$^-$ reduction of oxygen to water [1]. The enzyme contains 4 metal centers, 2 copper ions and 2 hemes, all of which are non-equivalent. One of the hemes, cytochrome a_3, has been shown [2,3] to be antiferromagnetically coupled to a cupric ion in the oxidized protein, and this coupled spin system apparently exhibits no electron paramagnetic resonance (EPR) spectrum.

Since cytochrome a_3 is known to bind certain exogenous ligands [1], this metal center has been extensively investigated through ligand binding studies. The addition of hydroxylamine to the oxidized protein or nitric oxide to the reduced form has been shown to give rise to a NO–ferrocytochrome c oxidase complex [4,5] which exhibits an EPR signal. The EPR spectrum of this complex is more isotropic than that observed for hemoproteins in the absence of NO, with $g_x = 2.09$, $g_z = 2.005$ and $g_y = 2.0$. It has been argued [6] that most of the unpaired electron spin density in NO–heme complexes reside in the d_{z^2} orbital of iron rather than in the π^* molecular orbital of the NO moiety. This description predicts super-hyperfine splitting on the g_z component of the EPR signal from magnetic nuclei axially ligated to cytochrome a_3. The observed superhyperfine pattern consists of 9 equally-spaced lines, and has been interpreted [5] in terms of the superposition of three sets of 3 lines arising from 2 non-equivalent nitrogens ($I = 1$) interacting with the unpaired electron. The larger of the 2 superhyperfine coupling constants

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(21.1 G) was assigned [5] to the nitrogen of bound nitric oxide and the smaller coupling constant (6.8 G) was assigned to a nitrogen on the endogenous axial ligand of cytochrome a_3. These assignments, while reasonable, need to be confirmed. The presence of an endogenous axial nitrogen ligand on cytochrome a_3, as well as the assignment of the coupling constants to 2 nitrogens, can be verified by investigating the EPR spectrum of ^{15}NO–ferrocytochrome c oxidase.

We report here the EPR spectrum of ^{15}NO–ferrocytochrome c oxidase. Our results verify that there is indeed an endogenous nitrogen axial ligand to the iron in cytochrome a_3, and that the assignment of the larger superhyperfine coupling constant to the NO nitrogen is correct. The possibility that the endogenous nitrogen ligand is due to histidine is discussed.

2. Materials and methods

Cytochrome c oxidase was isolated from beef heart mitochondria by the method in [7]. The heme a content was determined to be 8.9 nmol/mg protein by the pyridine hemochromagen assay [8]. The purified protein was dissolved in 0.5% Tween 20/50 mM Tris–HNO$_3$ (pH 7.4) to 56 mg protein/ml. Cytochrome c (Sigma type VI) was dissolved in 50 mM Tris–HNO$_3$ (pH 7.4) to 1 mM. All work with the proteins was carried out at 4°C or below. The protein–NO samples were prepared by first anaerobically reducing the proteins with dithionite, and then adding ^{15}NO (Prochem 99.21% ^{15}N) or ^{14}NO (Matheson) to the evacuated reduced protein samples to 0.7–0.9 atmospheres. These samples were allowed to equilibrate at 4°C for 30 min before being frozen at 77 K. The EPR spectra were recorded on a Varian E-Line Century

Series X-Band EPR Spectrometer. All spectra were
recorded at 85 K.

3. Results and discussion

The EPR spectra of ^{14}NO- and ^{15}NO-bound ferro-
cytochrome c oxidase are shown in fig.1 and 2,
respectively. The ^{14}NO—ferrocytochrome c oxidase
spectrum exhibits rhombic symmetry with $g_x = 2.091$,
$g_z = 2.006$, $g_y = 2.0$, and a 9 line superhyperfine
pattern on g_z with a splitting of 6.7 G, in agreement
with the results in [5]. The ^{15}NO-bound protein
exhibits an EPR spectrum with g values identical to
those of the ^{14}NO-bound species, but with a super-
hyperfine pattern of 2 sets of 3 lines. This pattern is
consistent with the presence of one ^{14}N and one ^{15}N
nitrogen axially bound to cytochrome a_3 with a
28.2 G splitting for the ^{15}N and a 6.8 G splitting for
the ^{14}N ligand. The observed increase of the larger
superhyperfine splitting from 20.3 G to 28.2 G upon
substitution of ^{15}NO for ^{14}NO is expected on the
basis of the relative magnetogyric ratios of the 2
nitrogen isotopes. These observations verify the
assignment [5] of the larger superhyperfine coupling
constant to the nitrogen of NO, and the smaller
superhyperfine coupling constant to the nitrogen of
the endogenous axial ligand of cytochrome a_3 in
cytochrome c oxidase.

The fifth nitrogen ligand to cytochrome a_3 could

CYTOCHROME $\underline{c}$ OXIDASE – ^{14}NO

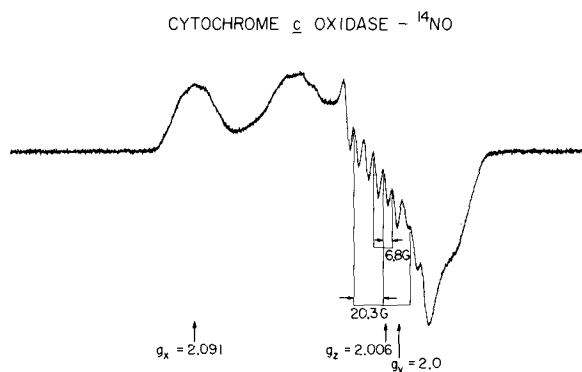$g_x = 2.091$ $g_z = 2.006$ $g_y = 2.0$ 6.8G   20.3G

Fig.1. The EPR spectrum of ^{14}NO–ferrocytochrome c oxidase.
The EPR spectra were recorded with 30 mw of microwave
power and at a microwave frequency of 9.154 GHz. The scan
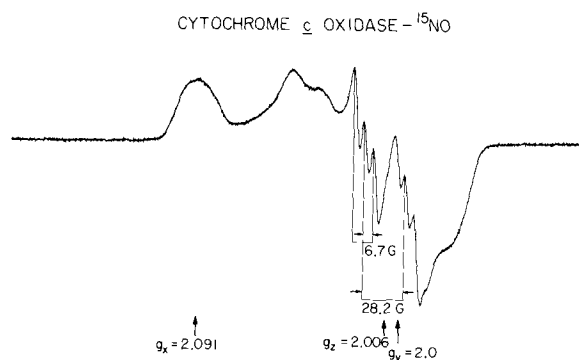time was 50 G/min and the modulation amplitude was 2 G.

CYTOCHROME $\underline{c}$ OXIDASE – ^{15}NO

$g_x = 2.091$ $g_z = 2.006$ $g_y = 2.0$ 6.7G   28.2 G

Fig.2. The EPR spectrum of ^{15}NO—ferrocytochrome c oxidase.

be contributed by any of a number of amino acids,
for example histidine, arginine, peptide nitrogen and
lysine. These potential fifth ligands vary greatly in
their π-bonding capabilities, with histidine being a
strong π-bonding ligand, and the remaining 3 ligands
being predominantly σ-bonding. In this regard, the g
values and nitrogen superhyperfine splittings of the
EPR signals of NO-bound hemin have been shown
[6] to be dependent on the π-bonding capability of the
axially-bound nitrogen ligand opposite NO. With non-
π-bonding ligands, such as amines, the EPR spectra
exhibit axial symmetry, with $g_x = g_y = 2.07$ and
$g_z = 2.008$. Also, the observed superhyperfine splitting
attributed to the bound ^{14}NO nitrogen is typically
16 G, and no superhyperfine splitting is resolved from
the nitrogen ligand opposite NO. In contrast, with
strong π-bonding ligands, such as pyridine, the EPR
spectra exhibit rhombic symmetry, with g values and
superhyperfine splittings similar to those of ^{14}NO-
bound ferrocytochrome c oxidase. This result suggests
that the endogenous axial ligand of cytochrome a_3 is
a strong π-bonding ligand, with histidine being the
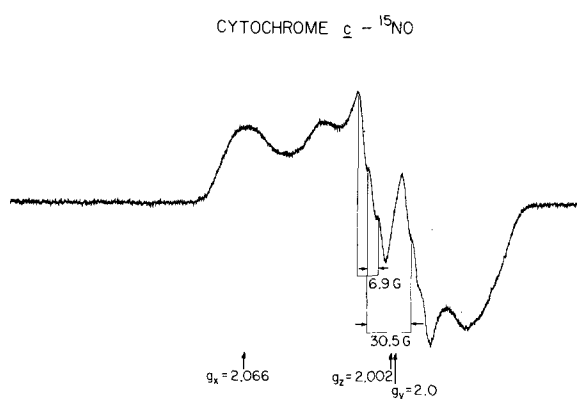most likely candidate [5].

Histidine is known to be an endogenous axial ligand
to iron in the hemoproteins hemoglobin and cyto-
chrome c. The EPR spectra of ^{14}NO- and ^{15}NO-bound
ferrocytochrome c [6,9] and ferrohemoglobin [10]
have been reported, and can be compared with the
EPR spectra of NO-bound ferrocytochrome c oxidase.
Figure 3 and 4 depict the EPR spectra of ^{14}NO- and
^{15}NO-bound ferrocytochrome c, which can be seen to
be similar to the corresponding spectra for NO-bound
cytochrome c oxidase. These similarities in the EPR

Fig.3. The EPR spectrum of ^{14}NO-ferrocytochrome c.
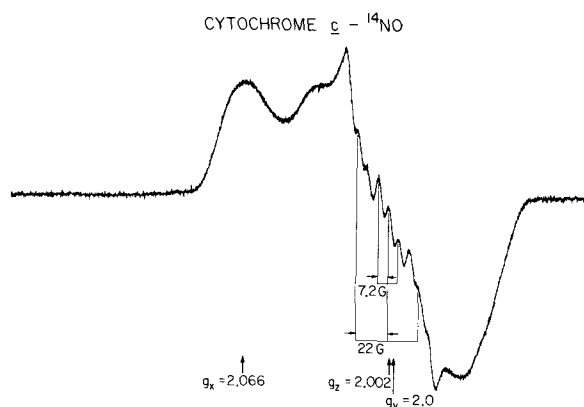
Fig.4. The EPR spectrum of ^{15}NO—ferrocytochrome c.

spectra of NO-bound ferrocytochrome c and NO-bound ferrocytochrome c oxidase have led to the suggestion [5] that the endogenous axial ligand to cytochrome a_3 in cytochrome c oxidase is also a histidine. However, other nitrogen ligands cannot be unequivocally ruled out on the basis of the available EPR data. EPR studies of ^{15}N-histidine incorporated cytochrome c oxidase would, however, definitely confirm the presence of an endogenous axial histidine ligand to cytochrome a_3. Efforts to this end are currently underway in our laboratory.

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