

Histidine Is the Axial Ligand to Cytochrome a_3 in Cytochrome c Oxidase*

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The nitric oxide-bound complexes of reduced yeast cytochrome c oxidase incorporated with $[1,3-^{15}\text{N}_2]$ histidine have been investigated by EPR spectroscopy. The results of this study have allowed the unambiguous identification of histidine as the endogenous axial ligand to cytochrome a_3 .

There has been considerable recent interest in the structure of the cytochrome a_3 - Cu_{a_3} site in cytochrome c oxidase because of the direct involvement of this binuclear center in the 4-electron reduction of molecular oxygen to water (1) in the final step of cellular respiration. A body of data is accumulating on the structure of this site (1-3). There is now ample evidence that cytochrome a_3 is a high spin heme iron in both the oxidized and reduced forms of the enzyme, suggesting that the heme is five-coordinate or that the sixth position is occupied by a weak ligand which is readily displaced by exogenous ligands. However, magnetic susceptibility data (4) indicate that cytochrome a_3 is strongly antiferromagnetically coupled to Cu_{a_3} in the oxidized enzyme to give a $S = 2$ ground state which is EPR-silent. A number of proposals has emerged on the nature of the ligand mediating this antiferromagnetic coupling between the two metal centers. One of these proposals (5) involves a bridging imidazole from a histidine residue. An endogenous nitrogen atom is indeed implicated in NO-binding studies (6, 7) of ferrocycytochrome a_3 in the reduced enzyme. While the EPR data suggest that this nitrogen may be part of a strong π -donating ligand (8), possibly an imidazole from the histidine residue (6), its identity has not been unambiguously determined.

The study described here is to identify the axial ligand of cytochrome a_3 . One approach to this problem is to prepare cytochrome c oxidase with ^{15}N -labeled amino acids and undertake NO-binding studies to the ferrocycytochrome a_3 in the labeled enzyme. Inasmuch as an imidazole of histidine seems

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to be the most likely candidate for the fifth ligand of the cytochrome a_3 , we have incorporated ^{15}N -labeled histidine into yeast cytochrome c oxidase. The results of this work reveal that the axial ligand is indeed a histidine residue in the yeast enzyme.

MATERIALS AND METHODS

The yeast strain *Saccharomyces cerevisiae* D273-10B was mutagenized by treatment with ethylmethanesulfonate and a nonpetite histidine auxotroph was isolated and purified by standard techniques (9). This histidine auxotroph was grown in a 350-liter fermentor in the following media: yeast nitrogen base (without amino acids) media (10); uracil and adenine, 11 g each; all L-amino acids except histidine, 17.5 g; DL- $[1,3-^{15}\text{N}_2]$ histidine, 95% ^{15}N -labeled in both ring positions (Vebberlin-Chemie, Germany), 2 g; galactose, 1%; penicillin, 11 g; streptomycin, 17 g. The yield of wet yeast cells was about 300 g, of which less than 0.004% had reverted to wild type.

Yeast mitochondria were isolated from the yeast cells by the method of Tzagoloff (11), as modified by Shakespeare and Mahler (12). The mitochondrial pellet was resuspended in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 70 mM sucrose, 110 mM mannitol, 1 mM EDTA, pH 7.4, and centrifuged at $100,000 \times g$. The resultant pellet was then resuspended in the same buffer to a protein concentration of 40-60 mg/ml. This concentrated mitochondrial suspension was used for subsequent EPR experiments. The wild type D273-10B yeast mitochondria were obtained precisely as described above except that no labeled histidine was added to the fermentor.

Beef heart cytochrome c oxidase was isolated by the procedure of Hartzell and Beinert (13). The purified protein (8.5 nmol of heme a /mg of protein) was dissolved in 0.5% Tween 20, 50 mM Tris/ HNO_3 , pH 7.4, to a protein concentration of 37 mg/ml.

The EPR samples were prepared by the addition of *p*-phenylenediamine and ascorbate to the anaerobic samples in EPR tubes to final concentrations of 2 and 20 mM, respectively. The NO-bound species were generated by the addition of either $\text{Na}^{14}\text{NO}_2$ or $\text{Na}^{15}\text{NO}_2$ (Prochem, 99% ^{15}N) to these samples (3) to a final concentration of about 20 mM. It has been found that the addition of NO_2^- to *p*-phenylenediamine- and ascorbate-reduced mitochondria¹ or the purified reduced (3) protein results in the observation of EPR signals from the NO-bound ferrocycytochrome a_3 of the enzyme almost exclusively. Samples of the reduced oxidase, or reduced mitochondrial suspensions, were allowed to incubate with NO_2^- for 30 min at 277 K under argon before being frozen at 77 K. The EPR spectra were recorded on a Varian E-line Century Series X-Band EPR spectrometer.

All of the yeast NO-bound spectra were recorded on mitochondrial suspensions. Since the concentration of cytochrome oxidase in the yeast mitochondrial samples was considerably lower than that in the purified beef heart cytochrome oxidase samples, it was necessary to collect multiple scans of the mitochondrial samples to obtain comparable signal to noise. In addition, background cavity signals plus small mitochondrial iron-sulfur protein signals were removed by subtracting 10 scans of reduced mitochondria from 10 scans of a sample containing reduced mitochondria plus NO_2^- on a Spex SCAMP SC-32 data processor.

RESULTS AND DISCUSSION

The binding of NO to ferrous hemoproteins is well known (14). In the case of fully reduced cytochrome c oxidase, NO binds to cytochrome a_3 to produce an NO-ferrocycytochrome c oxidase complex, which exhibits an EPR signal rich in hyperfine structure. The EPR spectra of ^{14}NO - and ^{15}NO -bound beef heart ferrocycytochrome c oxidase are shown in Fig. 1. When ^{14}NO is bound to ferrocycytochrome a_3 , the EPR signal of the complex exhibits a nine-line hyperfine pattern, which can be interpreted in terms of the superposition of three sets

¹ C. Martin and S. I. Chan, unpublished results.

of three lines arising from two nonequivalent nitrogens ($I = 1$) interacting with the unpaired electron (6, 7). The larger of the two hyperfine coupling constants is 20.3 G and the smaller 6.8 G. When ^{15}NO is used in this experiment, the ^{15}NO -bound protein exhibits an EPR spectrum with g values identical with those of the ^{14}NO -bound species, but with a hyperfine pattern consisting of two sets of three lines (7). This pattern is con-

sistent with the presence of one ^{14}N and one ^{15}N nitrogen bound axially 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 hyperfine 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 two nitrogen isotopes. These observations allow one to assign the larger of the hyperfine coupling constants to the nitrogen of the bound NO and the smaller coupling constant to a nitrogen on an endogenous axial ligand of cytochrome a_3 .

In Fig. 2, the EPR spectra of the normal NO-bound yeast protein are compared with those from the purified beef heart protein. Aside from the differences in signal to noise, the EPR parameters for the NO-bound beef heart and yeast proteins are essentially identical.

The EPR spectra of ^{14}NO - and ^{15}NO -bound $[1,3-^{15}\text{N}_2]$ histidine-substituted yeast cytochrome *c* oxidase are shown in Fig. 3. In contrast to the $[^{14}\text{N}]$ histidine NO-bound protein spectra, the hyperfine patterns in the $[1,3-^{15}\text{N}_2]$ histidine NO-bound protein have been altered. The $[1,3-^{15}\text{N}_2]$ histidine ^{15}NO -bound protein hyperfine pattern consists of two sets of doublets, with a ^{15}NO nitrogen splitting of 27.5 G and a splitting of about 12 G for the $[^{15}\text{N}]$ nitrogen of histidine. The $[1,3-^{15}\text{N}_2]$ histidine ^{14}NO -bound protein hyperfine pattern consists of three sets of doublets, with splittings of 21 G and 10.2 G for the ^{14}NO and histidine $[^{15}\text{N}]$ nitrogens, respectively. Thus, the substitution of $[1,3-^{15}\text{N}_2]$ histidine for $[^{14}\text{N}]$ histidine in cytochrome *c* oxidase results in the involvement of a ($I = 1/2$) ^{15}N nucleus rather than a ($I = 1$) ^{14}N nucleus in the NO-bound ferrocyclochrome a_3 EPR signal. The increase of the smaller hyperfine splitting from about 7 G to 10 G is expected on the basis of the relative magnetogyric ratios of the two nitrogen isotopes.

In summary, NO binding studies with $[1,3-^{15}\text{N}_2]$ histidine-substituted cytochrome *c* oxidase have allowed the unequivocal identification of histidine as the endogenous fifth ligand to cytochrome a_3 in the yeast enzyme. Although this study does not address the question of the imidazole bridge, recent work from our laboratory (2, 3) has established that this imidazole does not bridge the two metal centers at the cytochrome a_3 -Cu $_a$ site. Thus, the histidine imidazole is the distal axial ligand of cytochrome a_3 *vis-à-vis* Cu $_a$.

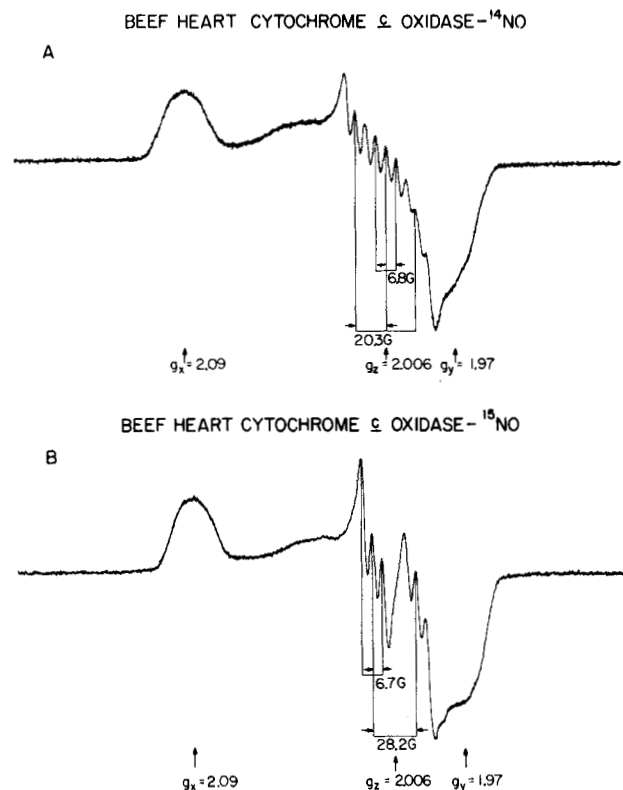


FIG. 1. EPR spectra of NO-bound reduced beef heart cytochrome *c* oxidase with ^{14}NO (A) and ^{15}NO (B), prepared by the addition of either $^{14}\text{NO}_2^-$ or $^{15}\text{NO}_2^-$ to 0.16 mM cytochrome *c* oxidase. Conditions: temperature, 50 K; microwave power, 5 milliwatts; modulation amplitude, 2 G; microwave frequency, 9.23 GHz.

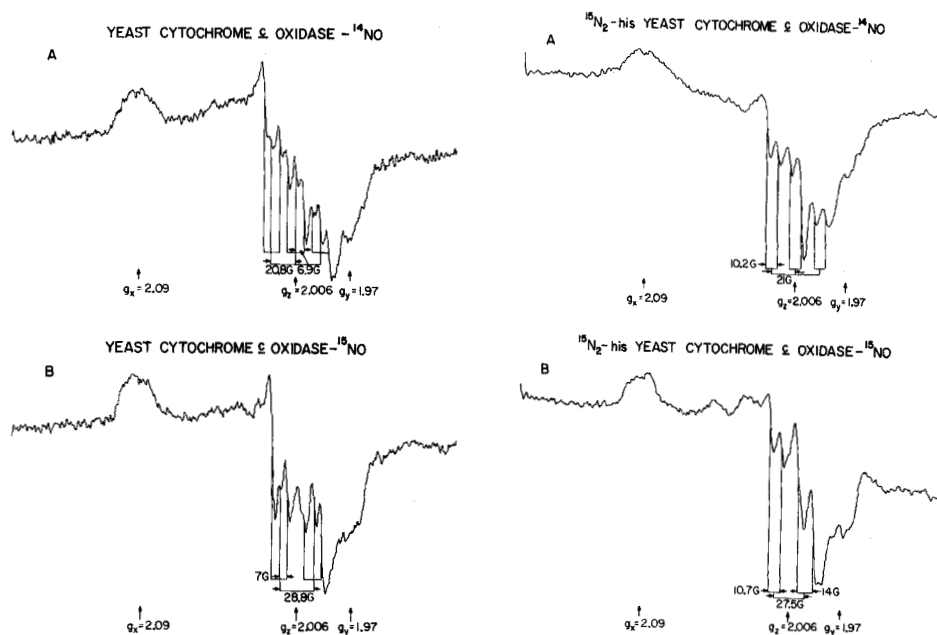


FIG. 2 (left). EPR spectra of NO-bound reduced yeast cytochrome *c* oxidase with ^{14}NO (A) and ^{15}NO (B). The samples were prepared by the addition of either $^{14}\text{NO}_2^-$ or $^{15}\text{NO}_2^-$ to *p*-phenylenediamine- and ascorbate-reduced yeast mitochondria. Conditions: temperature, 30 K; microwave power, 2 milliwatts; modulation amplitude, 2 G; microwave frequency, 9.21 GHz.

FIG. 3 (right). EPR spectra of NO-bound $[1,3-^{15}\text{N}_2]$ histidine-substituted reduced yeast cytochrome *c* oxidase with ^{14}NO (A) and ^{15}NO (B), prepared by the addition of either $^{14}\text{NO}_2^-$ or $^{15}\text{NO}_2^-$ to *p*-phenylenediamine- and ascorbate-reduced $[1,3-^{15}\text{N}_2]$ histidine-labeled yeast mitochondria. Conditions: temperature, 30 K; microwave power, 2 milliwatts; modulation amplitude, 4 G; microwave frequency, 9.21 GHz.

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