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The Identification of Histidine Ligands to Cytochrome *a* in Cytochrome *c* Oxidase*

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A histidine auxotroph of *Saccharomyces cerevisiae* has been used to metabolically incorporate [1,3-$^{15}N_2$] histidine into yeast cytochrome *c* oxidase. Electron nuclear double resonance (ENDOR) spectroscopy of cytochrome *a* in the [^{15}N]histidine-substituted enzyme reveals an ENDOR signal which can be assigned to hyperfine coupling of a histidine ^{15}N with the low-spin heme, thereby unambiguously identifying histidine as an axial ligand to this cytochrome. Comparison of this result with similar ENDOR data obtained on two ^{15}N-substituted bisimidazole model compounds, metmyoglobin-[^{15}N]imidazole and bis[^{15}N]imidazole tetraphenyl porphyrin, provides strong evidence for bisimidazole coordination in cytochrome *a*.

Cytochrome *a* is generally thought to be the primary acceptor of electrons in cytochrome *c* oxidase. It can accept electrons from cytochrome *c* and transfer them to the other metal centers of the protein (1, 2). Consistent with this function, the midpoint potential of cytochrome *a* is quite similar to that of cytochrome *c* (3, 4); this potential matching allows for a small loss in the total available redox energy at this initial electron transfer step. From cytochrome *a*, each electron is then transferred to dioxygen coordinated at the cytochrome a_3-Cu_B binuclear center. The electrons are generally assumed to be transferred to the oxygen reduction site via Cu_A, although recent evidence suggests that under certain conditions cytochrome *a* may also transfer electrons *directly* to this site (5).

The potential difference between cytochrome *c* and dioxygen, 42 kcal/mol of oxygen reduced (6), is quite substantial. Cytochrome oxidase converts some of this potential energy into an electrochemical proton gradient across the mitochondrial inner membrane (7). It has been proposed that cytochrome *a* is directly involved in proton pumping, coupling the electron transfer events at the site to the translocations of protons across the mitochondrial membrane (8). However, almost nothing is currently known about the mechanisms of electron transfer and/or proton pumping in cytochrome oxidase. It is clear that a knowledge of the structures of the involved metal centers is crucial for an understanding of the coupling between electron transfer and proton pumping in this important enzyme.

The cytochrome *a* site is known to consist of a low-spin iron coordinated by four in-plane nitrogen ligands from a heme a macrocycle and is presumably six-coordinate with two axial ligands. No direct evidence exists as to the identity of the (endogenous) axial ligands. Blumberg and Peisach (9) have compared the electron paramagnetic resonance spectral *g*-values of a large variety of low-spin heme model compounds and on the basis of these comparisons have argued for bisimidazole coordination in cytochrome *a*. Similar comparisons using optical and resonance Raman spectroscopies (10) and magnetic circular dichroism spectroscopy (11-14) have led investigators to the same conclusion. However, none of these studies provides direct information on the identities of the axial ligands to cytochrome *a*. Other studies have in fact noted that all of the spectral properties of cytochrome *a* site may not be satisfactorily simulated by model compounds (15, 16). In any case, to date no definitive evidence for either mono- or bisimidazole coordination of cytochrome *a* has been produced.

Recently we combined the unique capabilities of electron nuclear double resonance (ENDOR[1]) spectroscopy and the specific incorporation of isotopically substituted amino acids into yeast cytochrome *c* oxidase to identify the ligands to the Cu_A center in cytochrome oxidase (17). In this report, we use an analogous approach to identify the axial ligands to cytochrome *a*. We present ENDOR studies of native and [1,3-$^{15}N_2$]histidine-substituted yeast cytochrome oxidase which demonstrate conclusively the coordination of at least one histidine ligand to cytochrome *a* in cytochrome oxidase. Comparison of the observed ^{15}N hyperfine couplings with those from two well-characterized bisimidazole porphyrin model compounds offers strong evidence for the coordination of a second histidine imidazole as well.

MATERIALS AND METHODS

Preparation of Native and Isotopically Substituted Cytochrome Oxidase

All chemicals used in the enzyme purification were of enzyme grade when available; otherwise they were reagent grade. All the nutrients

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[1] The abbreviations used are: ENDOR, electron nuclear double resonance; [^{15}N]His, [1,3-$^{15}N_2$]histidine.

used in the growth of yeast such as vitamins, amino acids, and galactose were of the highest grades available from Sigma. The [1,3-$^{15}N_2$]histidine·HCl was 95% enriched in ^{15}N at both histidine ring positions and was obtained from Veb Berlin-Chemie, Berlin-Adlershof, West Germany. The per cent enrichment was verified shortly before incorporation into the yeast by natural abundance ^{13}C NMR (18).

Wild Type Yeast—The wild type *Saccharomyces cerevisiae* haploid strain D273-10B (mating type *α*) was used in the growth of yeast for the isolation of unsubstituted (native) protein. This strain has been shown to respire efficiently on the nonrepressive carbon source galactose (*i.e.* it contains the GAL^+ trait) and produces good quantities of mitochondria.

Histidine Auxotrophs—For the preparation of the [1,3-$^{15}N_2$]histidine-substituted protein, the *S. cerevisiae* auxotroph haploid strains designated SS328 (*α* ura_3-52, GAL, SUC2, δhis 3, D200, lys2-801°, ade2-101°) and SS330 (a ura_3-52, GAL, SUC2, δhis 3, D200, tyr1, ade2-101°) were obtained from Drs. Stu Scherer and Carl Parker (Caltech). These two haploid strains were crossed just before use so that subsequent growth was predominantly in the diploid form. These strains were found to be ideally suited for the preparation of [^{15}N] His-substituted protein in that the histidine mutation in these strains is a gene deletion rather than a single base substitution; consequently, revertant levels were always well below the detection limit (less than 0.001%).

Large-scale Growth of Yeast—The growth of yeast cells for the isolation of native (unsubstituted) and [^{15}N]His-substituted protein was carried out in a 360-liter fermentor, as has been described previously (17, 18), except that the media for the growth of the [^{15}N] His-substituted yeast contained the following specifically added amino acids: 5 g each of Ser, Met, Thr, Trp, Tyr, Phe, Asn, Glu, Arg; 20 g of Gly; 50 g of Lys; and 4.0 g of DL-[1,3-$^{15}N_2$]histidine·HCl (95% ^{15}N at both imidazole ring positions). Cells were allowed to grow to a density of 2.9×10^7 cells/ml. At harvest, the cell density of revertants was below the level of detection (0.001%).

Preparation of Yeast Cytochrome Oxidase—Yeast mitochondria were isolated according to the procedure of George-Nascimento *et al.* (19), except that the buffer used during the Dyno-Mill cell disruption was 0.4 M in sucrose. This procedure resulted in the breakage of at least 80% of the yeast cells. Yeast cytochrome oxidase was isolated from the mitochondria and purified as described previously (17). The final protein was suspended in 0.5% Tween 20, 20 mM Tris, pH 7.4. Protein concentration was typically 0.1 mM in 0.2–0.3-ml sample volumes.

Preparation of Beef Heart Cytochrome Oxidase—Cytochrome oxidase from beef heart was prepared by the method of Yu *et al.* (20) and was a phospholipid "sufficient" sample. The protein concentration was 165 mg/ml (with 11 nmol of heme a/mg of protein). The protein was suspended in 1.0% sodium cholate, 50 mM potassium phosphate, pH 7.4.

Preparation of Model Compounds

Myoglobin-Imidazole—Sperm whale metmyoglobin was purchased from Sigma, chromatographed on Whatman DE52, and dissolved to a protein concentration of 5 mM in 1:1 (v/v) glycerol/water, 50 mM potassium phosphate, pH 7.4. Imidazole was added to a concentration of 40 mM to form the six-coordinate metmyoglobin-imidazole complex. Unsubstituted imidazole was purchased from Sigma and recrystallized from benzene-ethanol before use. The [^{15}N]imidazole (99% ^{15}N at both ring positions) was obtained from Stohler Isotopes and recrystallized from benzene-ethanol before use.

Bisimidazole Tetraphenyl Porphyrin—The bisimidazole Fe(III)-tetraphenyl porphyrin complexes were 3 mM in tetraphenyl porphyrin (Strem) and 40 mM in imidazole (as above) and were dissolved in 1:1 $CDCl_3/CD_2Cl_2$.

Spectroscopy

EPR Spectroscopy—EPR spectra were recorded on a Varian E-Line Century Series X-band spectrometer equipped with an Air Products Heli-Trans low temperature controller. Data were collected and stored on a PDP8/A (Digital Equipment Corp.) microcomputer interfaced to the spectrometer. Instrumental conditions are given in the figures.

ENDOR Spectroscopy—ENDOR spectra were recorded at State University of New York at Albany on equipment and, except as noted in the figures, under the conditions described previously (21–23).

RESULTS

Characterization of Isotopically Substituted Yeast Cytochrome Oxidase

EPR Spectroscopy—The EPR spectra of native (unsubstituted) yeast and [1,3-$^{15}N_2$]histidine-substituted yeast cytochrome oxidase are compared in Fig. 1. The spectra are virtually identical and, in particular, show very little high-spin heme or adventitious copper. Also indicated in Fig. 1 are the positions of ENDOR observation for the cytochrome *a* (*g* = 2.24) and Cu_A (*g* = 2.04) studies. Note that the position of ENDOR observation for the cytochrome *a* ENDOR studies is in a region of the EPR spectrum in which there should be little or no contribution from Cu_A.

ENDOR Spectroscopy—The spectra observed at *g* = 2.04 for native and [^{15}N]His-substituted yeast cytochrome oxidase are shown in Fig. 2. They arise almost completely from the

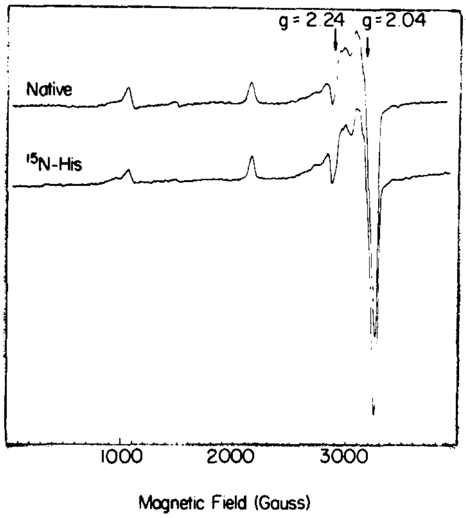

FIG. 1. **EPR spectra of native and [^{15}N]His-substituted yeast cytochrome oxidase showing both the Cu_A and cytochrome *a* signals.** Conditions: temperature, 15 K; microwave power, 10 microwatts; field modulation, 16 G; microwave frequency, 9.179 GHz.

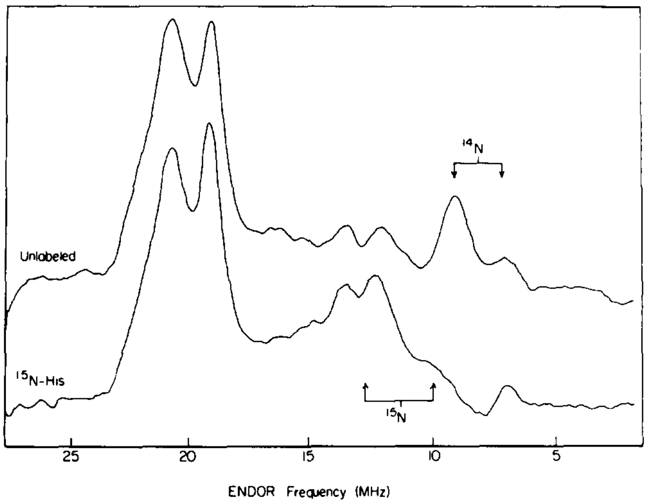

FIG. 2. **ENDOR spectra of Cu_A (observed at *g* = 2.04) in native and [^{15}N]His-substituted yeast cytochrome oxidase.** Conditions: temperature, 2.1 K; microwave power, 10 microwatts; microwave frequency, 9.03 (*unlabeled*) and 9.08 (*^{15}N-His*) GHz; field modulation, 4.0 G; sweep rate, 5.2 MHz/s; instrumental time constant, 0.02 s.

Cu_A center in cytochrome oxidase. These data show conclusively that histidine is a ligand to Cu_A. They also serve to demonstrate that ^{15}N has been successfully incorporated into the [^{15}N]His-substituted sample.

ENDOR Studies of Isotopically Substituted Model Compounds

Before presenting the ENDOR results for cytochrome *a* in [^{15}N]His-substituted cytochrome oxidase, we first compare the ENDOR spectra of two bisimidazole porphyrin model compounds substituted with [1,3-$^{15}N_2$]imidazole. Native myoglobin contains a five-coordinate ferric heme center, with an endogenous histidyl imidazole providing an axial nitrogen ligand and a second axial site open to coordination of exogenous ligands. Addition of imidazole to metmyoglobin converts the center to a low-spin bisimidazole heme iron. This exogenous imidazole ligand can easily be replaced by [^{15}N]imidazole to yield a species in which one imidazole is ^{15}N-substituted and the other is (naturally abundant) ^{14}N. Another low-spin porphyrin model compound which has been studied here is bisimidazole tetraphenyl porphyrin. This complex has been prepared with both axial imidazoles containing either ^{14}N (natural abundant) or ^{15}N (enriched) at the nitrogen position.

Myoglobin-Imidazole—The ENDOR spectrum of metmyoglobin coordinated by exogenous native imidazole (Fig. 3) contains several overlapping peaks in the low-frequency region between 1 and 5 MHz, as do the spectra of many other low-spin ferric heme systems. This spectrum was recorded near the middle *g*-value ($g = 2.26$) in order to obtain optimal signal intensity.

The corresponding ENDOR spectrum of metmyoglobin coordinated by exogenous [^{15}N]imidazole is also shown in Fig. 3. A new signal is observed at 5.66 MHz and must be a result of the ^{15}N substitution in imidazole. Thus, although the ^{14}N ENDOR signals in the spectrum of the *native* protein are difficult to assign to individual coordinating atoms, the new signal observed in the ^{15}N-substituted sample stands out and can be assigned with certainty to an axial imidazole nitrogen. Spectra recorded at *g*-values in the 2.26 (*g*-intermediate) to 1.52 (*g*-minimal) region show a ^{15}N ENDOR line with approximately the same frequency as that shown in Fig. 3; these *g*-values are in directions parallel to the heme plane. At the maximal *g*-value (2.90), the ^{15}N ENDOR line at 5.66 MHz disappears, and a weak ENDOR line occurs below 2 MHz.

For coupling to a single ^{15}N (or to two magnetically equivalent ^{15}Ns), two ENDOR signals should occur centered at one-half the ^{15}N hyperfine coupling and separated by twice the characteristic ^{15}N Zeeman frequency, $\nu(^{15}N)$. (Under the conditions of Fig. 3, $2\nu(^{15}N) = 2.48$ MHz.) Thus, for the ^{15}N signal observed in Fig. 3, a Zeeman partner is predicted to occur at either 8.14 or 3.18 MHz. Since no signals are found near 8 MHz in Fig. 3, we conclude that the Zeeman partner for the ^{15}N signal must occur at 3.18 MHz, but is obscured by the other signals in this region of the spectrum. A detailed comparison of the derivatives of the two spectra in Fig. 3 shows that there is indeed an inflection at 3.15 MHz from the ^{15}N-substituted imidazole but not the ^{14}N-compound. Thus, we assign a ^{15}N hyperfine interaction of 8.8 MHz for the exogenous axial imidazole nitrogen ligand.

Bisimidazole Tetraphenyl Porphyrin—A similar result is seen for native and ^{15}N-substituted bisimidazole tetraphenyl porphyrin. The ENDOR spectra of the native ^{14}N- and [^{15}N] imidazole-substituted forms are compared in Fig. 4. In the spectrum of the [^{15}N]imidazole-substituted sample, there is clearly a new signal at approximately 5.6 MHz attributable to coupling to an imidazole ring [^{15}N]nitrogen. The other non-imidazole signals observed between 2 and 5 MHz most likely arise from porphyrin ring nitrogens and appear very similar to those seen for the bisimidazole myoglobin samples.

ENDOR Comparison of Native and Isotopically Substituted Cytochrome a

The ENDOR spectrum of cytochrome *a* in native yeast cytochrome oxidase observed at $g = 2.24$ is shown in Fig. 5. At this *g*-value, the cytochrome *a* contribution to the ENDOR absorption spectrum is maximized whereas that from Cu_A is negligible. The general features of the spectrum are quite similar to those of unsubstituted myoglobin-imidazole and bisimidazole tetraphenyl porphyrin. In particular, there is a collection of unresolved signals in the low-frequency region between 1 and 5 MHz, presumably arising from equatorially coordinated heme ring nitrogens (22).

The ENDOR spectrum of [^{15}N]His-substituted yeast cytochrome oxidase at $g = 2.24$ is also shown in Fig. 5. Comparison of this spectrum with that of the native yeast protein reveals a new signal near 5.6 MHz in the spectrum of the ^{15}N-substituted protein. This result is analogous to those obtained

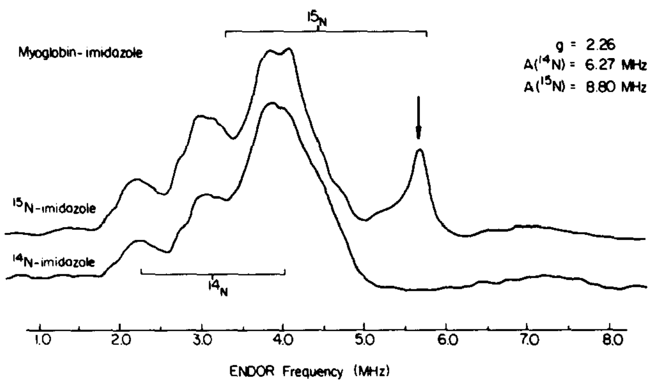

FIG. 3. **ENDOR spectra of native and [^{15}N]imidazole-substituted metmyoglobin-imidazole (observed at $g = 2.26$).** Conditions: temperature, 2.1 K; microwave power, 3.2 microwatts; microwave frequency, 9.09 GHz; field modulation, 2.0 G; sweep rate, 0.8 MHz/s; instrumental time constant, 0.05 s.

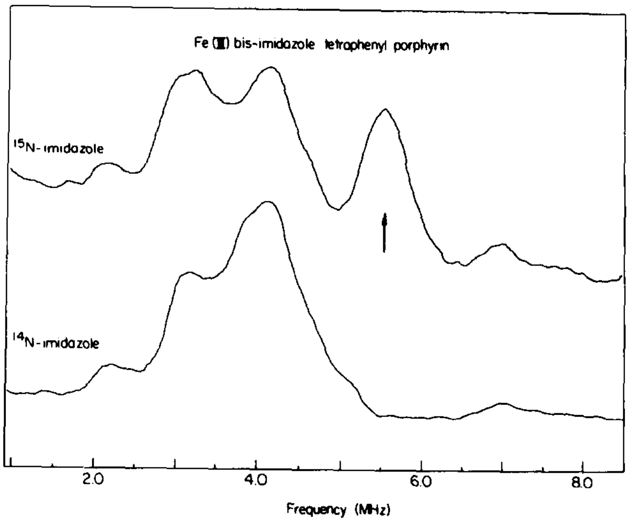

FIG. 4. **ENDOR spectra of native and [^{15}N]imidazole-substituted bisimidazole tetraphenyl porphyrin (observed at $g = 2.28$).** Conditions: temperature, 2.1 K; microwave power, 3.2 microwatts; microwave frequency, 9.13 (^{14}*N-imidazole*) and 9.17 (^{15}*N-imidazole*) GHz; field modulation, 2.0 G; sweep rate, 0.8 MHz/s; instrumental time constant, 0.05 s.

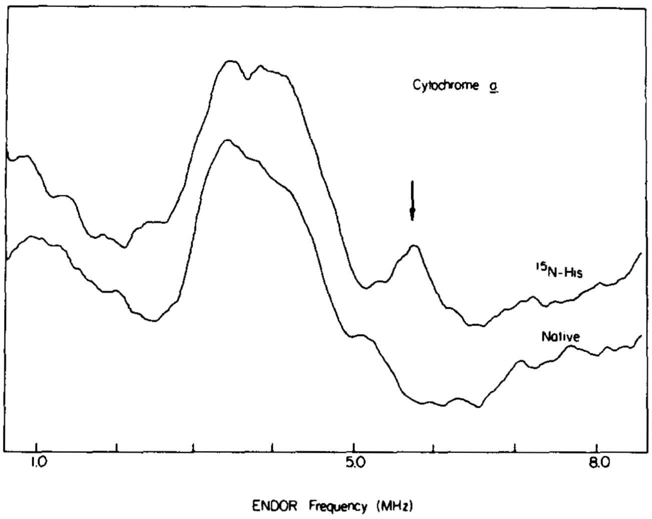

FIG. 5. **ENDOR spectra of cytochrome *a* in native and [^{15}N] His-substituted yeast cytochrome oxidase (observed at g = 2.24).** Conditions: temperature, 2.1 K; microwave power, 3.2 microwatts; microwave frequency, 9.03 (*native*) and 9.09 (*^{15}N-His*) GHz; field modulation, 2.0 G; sweep rate, 1.6 MHz/s; instrumental time constant, 0.05 s.

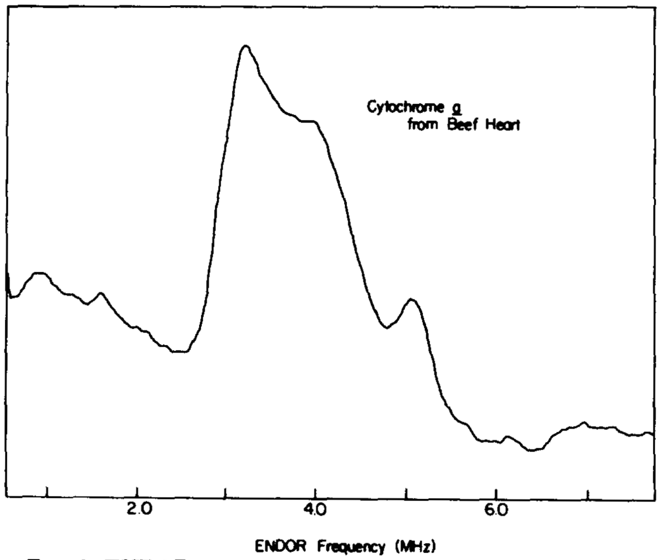

FIG. 6. **ENDOR spectrum of cytochrome *a* in beef heart cytochrome oxidase (observed at g = 2.26).** Conditions: temperature, 2.1 K; microwave power, 3.2 microwatts; microwave frequency, 9.09 GHz; field modulation, 2.0 G; sweep rate, 1.6 MHz/s; instrumental time constant, 0.02 s.

for the native and ^{15}N-substituted forms of bisimidazole myoglobin and for bisimidazole tetraphenyl porphyrin. The presence of this new signal clearly demonstrates the coordination of at least one histidyl imidazole nitrogen to cytochrome *a*. Moreover, the calculated ^{15}N hyperfine coupling in cytochrome *a*, assuming a ^{15}N ENDOR partner near 3.1 MHz, is approximately 8.8 MHz, the same as that observed in the myoglobin study and for bis[^{15}N]imidazole tetraphenyl porphyrin.

ENDOR Spectrum of Cytochrome a in the Beef Heart Protein

Due to the large g-value anisotropy in the EPR spectrum of cytochrome *a*, the EPR absorption intensity, and hence the ENDOR signal intensity, is an order to magnitude smaller for cytochrome *a* than for Cu_A. Consequently, it is difficult to obtain spectra with substantial signal-to-noise with the small amounts of yeast protein available. In an attempt to resolve some of the signals in the 1–5-MHz region of the ENDOR spectrum of cytochrome *a*, we examined a much larger (about five times greater) volume of cytochrome oxidase isolated from beef heart. The ENDOR spectrum of cytochrome *a* from the beef heart protein, shown in Fig. 6, is almost identical to that of the native yeast protein, although the spectrum for the former is somewhat better resolved because of the smaller spectrometer time constant used to record the spectrum.

The sharp peak at 5.0 MHz is not seen from the bisimidazole models studied here or with other low-spin ferric heme proteins (*e.g.* myoglobin CN^-, myoglobin N_3^-, cytochrome *c*) when studied at their intermediate g-value. A similar peak does occur from cytochrome b_5, which is known to have bisimidazole ligation, as well as from native yeast cytochrome oxidase. The peak is conceivably from [^{14}N]imidazole and in support of this, we note that it is significantly attenuated, if not missing, in the cytochrome *a* ENDOR spectrum of the [^{15}N]His-substituted yeast oxidase. Another possibility is that the signal originates from a heme [^{14}N]nitrogen whose hyperfine and quadrupole interactions are uniquely affected by bisimidazole ligation in these proteins.

The remaining features of the spectrum are not well resolved although they occur in a general-frequency region where we have obtained ENDOR from ^{14}N of the low-spin ferric heme nitrogens (21, 22). In the absence of isotopic substitution data for these signals, further assignment will not be attempted. ENDOR spectra of a frozen solution sample taken at the intermediate g-value of a rhombic EPR signal often yield features that are not well resolved. This happens because there will be many angular orientations of the molecule that can have the intermediate g-value, and different hyperfine and quadrupole couplings may be associated with these orientations. Evidently, the same hyperfine coupling is associated with enough of these orientations (no quadrupole coupling!) for the histidyl imidazole ^{15}Ns to yield a fairly well-resolved ENDOR resonance in the [^{15}N]His-substituted derivatives.

DISCUSSION

The coordination environments of the metal centers in cytochrome *c* oxidase are almost certainly the same in both the yeast and beef heart forms of the enzyme. The ENDOR results presented here for cytochrome *a* in native cytochrome oxidase from both species are indistinguishable within the resolution obtainable. The ability, in the yeast protein, to specifically incorporate histidine substituted with ^{15}N at the two imidazole ring nitrogen positions provides an opportunity to directly probe the axial coordination of cytochrome *a*, specifically to determine whether histidine is an axial ligand to this heme iron center in cytochrome oxidase.

The finding of a new ENDOR resonance in the spectrum of cytochrome *a* in [1,3-$^{15}N_2$]histidine-substituted yeast cytochrome oxidase which is not present in the corresponding spectrum of the native enzyme is unambiguous proof that histidine provides at least one axial nitrogen ligand to cytochrome *a*. The strength of the hyperfine interaction for this ^{15}N coupling (A = 8.8 MHz) is identical to the ^{15}N coupling observed for the ^{15}N-substituted myoglobin-imidazole complex, where the exogenous axial imidazole ligand is ^{15}N-substituted at both ring nitrogens. The coupling in cytochrome *a* is also very similar to the ^{15}N coupling observed for bis[^{15}N]imidazole tetraphenyl porphyrin. These comparisons strongly suggest that cytochrome *a* is also bisimidazole in coordination. We point out that the observation of only one new resonance in the ENDOR spectrum of [^{15}N]His-substituted cytochrome *a*, relative to that of the unsubstituted center, does not preclude the existence of a second histidine ligand to cytochrome *a*. In fact, the possibility that the resonances of both axial ligands are coincident would reflect a degree of symmetry in the coordination environment near the axial ligands.

CONCLUSION

The incorporation of [^{15}N]His into yeast cytochrome oxidase has provided unambiguous proof of the coordination of at least one histidine imidazole nitrogen as an axial ligand to cytochrome *a*. The similarity of the ^{15}N hyperfine coupling reported here for [^{15}N]His-substituted cytochrome *a* to the ^{15}N hyperfine couplings observed for the axial imidazoles in two bisimidazole porphyrin model compounds provides strong evidence in support of bisimidazole coordination with two axial histidine ligands to cytochrome *a*.

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