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Direct Evidence for a Tyrosine Radical in the Reaction of Cytochrome *c* Oxidase with Hydrogen Peroxide[†]

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ABSTRACT: Cytochrome *c* oxidase (COX) catalyzes the reduction of oxygen to water, a process which is accompanied by the pumping of four protons across the membrane. Elucidation of the structures of intermediates in these processes is crucial for understanding the mechanism of oxygen reduction. In the work presented here, the reaction of H_2O_2 with the fully oxidized protein at pH 6.0 has been investigated with electron paramagnetic resonance (EPR) spectroscopy. The results reveal an EPR signal with partially resolved hyperfine structure typical of an organic radical. The yield of this radical based on comparison with other paramagnetic centers in COX was ~20%. Recent crystallographic data have shown that one of the Cu_B ligands, His 276 (in the bacterial case), is cross-linked to Tyr 280 and that this cross-linked tyrosine is ideally positioned to participate in dioxygen activation. Here selectively deuterated tyrosine has been incorporated into the protein, and a drastic change in the line shape of the EPR signal observed above has been detected. This would suggest that the observed EPR signal does indeed arise from a tyrosine radical species. It would seem also quite possible that this radical is an intermediate in the mechanism of oxygen reduction.

Cytochrome *c* oxidase (COX;[1] for recent reviews, see refs *1* and *2*) is the terminal component of the respiratory chain of mitochondria and many aerobic bacteria. It catalyzes electron transfer from cytochrome *c* to molecular oxygen, thereby reducing the latter to water. The redox reaction is coupled to proton pumping across the inner mitochondrial or bacterial membrane (*3*) so that the net reaction can be written as

$$4\text{cyt } c^{2+} + O_2 + 8H^+_i \rightarrow 4\text{cyt } c^{3+} + 2H_2O + 4H^+_o$$

with H^+_i denoting protons taken up from the inner phase (the cytoplasm or mitochondrial matrix) and H^+_o referring to protons released into the outer phase (the periplasm or mitochondrial intermembrane space).

During the catalytic cycle, oxygen binds to the reduced heme a_3-Cu_B binuclear center forming the so-called compound **A** (*4*). In compound **A**, like in oxyhemoglobin, the oxygen binds to the heme in a relaxed, end-on configuration (*5*). Electronic reorganization then produces state **P**, which

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[1] Abbreviations: A_{iso}, isotropic hyperfine coupling; COX, cytochrome *c* oxidase; cw, continuous wave; DPPH, α,α′-diphenyl-β-picrylhydrazyl; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance; FTIR, Fourier transform infrared spectroscopy; hf, hyperfine; hfcs, hyperfine coupling constants; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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was believed to be a ferric−cupric peroxy species (*6*, *7*). Further electron input via Cu_A and heme *a* yields an oxoferryl−cupric **F** species (*8*). After transfer of the fourth electron from cytochrome *c* to the binuclear center and formation of the second water molecule, the oxidized enzyme is regenerated.

Compound **P** can also be obtained by addition of low concentrations of hydrogen peroxide to the oxidized enzyme (*9*−*11*), or in reversed-flow experiments as the result of a two-electron reversal (*6*, *12*). Raman spectra of the **P** state produced in the reaction with H_2O_2 (*13*) and in the reactions of the mixed-valence (*14*) and fully reduced (*15*) enzyme with O_2 exhibited a signal at 804 cm^{-1} that was assigned to a ν(Fe═O) mode, indicating that the O−O bond is already broken at this stage of the oxygen reduction cycle. The reductive cleavage of this bond, however, requires four electrons, and only three [two electrons from $Fe_{a3}(II) \rightarrow Fe_{a3}(IV)$, one electron from $Cu_B(I) \rightarrow Cu_B(II)$] can normally be provided by the metal centers. To account for the fourth electron, oxidation of Fe(IV) to Fe(V) (*14*) or Cu(II) to Cu(III) (*11*) has been proposed. The missing electron could also be donated, like in ascorbate oxidase (*16*) or catalase (*17*, *18*), by the heme macrocycle, but so far neither optical (*10*, *11*, *19*) nor Raman (*13*) spectroscopy provides evidence of formation of a porphyrin π-cation radical. The fourth possibility is oxidation of an amino acid to form a radical, as has been observed in the reactions of prostaglandin H synthase (*20*) and cytochrome *c* peroxidase (*21*).

The crystal structures of the cytochrome *c* oxidases from *Paracoccus denitrificans* (*22*) and bovine heart (*23*) show that a conserved tyrosine residue close to the binuclear center (Tyr 280 in *P. denitrificans*) is covalently cross-linked to a histidine (His 276) which is a ligand to the Cu_B. It has been speculated that this tyrosine could be the source of the fourth electron required for O−O bond cleavage (*14*, *22*, *24*−*26*), but so far no experimental results supporting the formation of a tyrosine radical in the course of the cytochrome *c* oxidase reaction have been reported.

Here we present direct evidence for the formation of a tyrosine radical generated in the reaction of *P. denitrificans* cytochrome *c* oxidase with hydrogen peroxide. This species was unequivocally assigned to a tyrosine radical using electron paramagnetic resonance (EPR) spectroscopy in combination with specific ^2H labeling.

MATERIALS AND METHODS

P. denitrificans (ATCC13543; *27*) cells were grown in succinate medium as described previously (*28*), except that no manganese was present in the medium. Cytochrome *c* oxidase was isolated as described previously (*29*). For the generation of [$^2H_4$]tyrosine-labeled cytochrome *c* oxidase, the culture medium was supplemented with 160 mg of [2,3,5,6-^2H]tyrosine (Campro Scientific, Emmerich, Germany) per liter. This resulted in a labeling efficiency of at least 90%, as judged by EPR and FTIR spectroscopy (P. Hellwig, J. Behr, H. Michel, and W. Mäntele, unpublished experiments).

The COX concentration was 200 μM in 0.1 M Mes-KOH (pH 6.0) and 0.05% *n*-dodecyl-β-D-maltopyranoside. The reaction was initiated by addition of 1 molar equiv of H_2O_2 at 4 °C, and the samples were immediately transferred to the standard Suprasil quartz EPR tubes (outside diameter of 3 or 4 mm) and frozen in liquid nitrogen. Cyanide-inhibited COX was formed by incubation with potassium cyanide (5 mM) at 4 °C for several hours. The reaction with H_2O_2 was as described above.

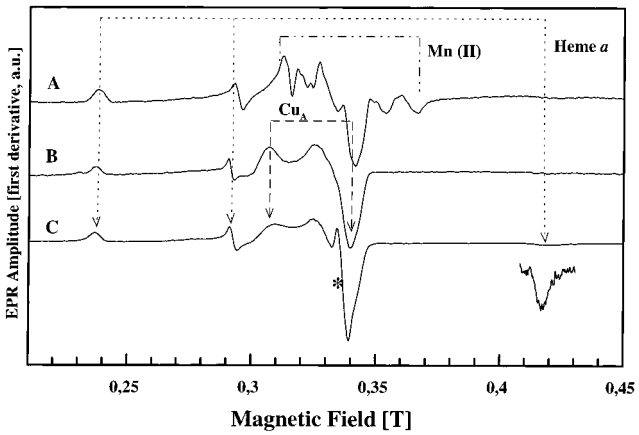

FIGURE 1: (A) EPR spectrum of native cytochrome *c* oxidase isolated from *P. denitrificans*. (B) EPR spectrum of native COX grown in Mn(II)-depleted medium. (C) EPR spectrum of COX in Mn(II)-depleted medium and treated with H_2O_2. In trace C, the radical EPR signal is denoted with an asterisk and the third component of the heme *a* EPR signal is enlarged for illustration purposes. The experimental conditions were as follows: microwave power, 20 mW; field modulation, 12.5 kHz; amplitude, ±0.5 mT peak to peak; and $T = 20$ K; except for trace C, microwave power, 0.1 mW; and $T = 10$ K.

X-Band continuous-wave (cw) and pulsed-EPR spectra of the radical EPR signal (Figure 2) were recorded on a Bruker E580 ELE*X*SYS spectrometer. A standard dielectric ring Bruker EPR cavity (MD 5 W1) was used which was equipped with an Oxford helium (CF935) cryostat. The microwave frequency and magnetic field were measured using the Bruker internal frequency counter and field controller, respectively. In addition, some control cw-EPR spectra of Mn(II), Cu_A, and heme *a* were recorded on a Bruker ESP300 spectrometer using a standard rectangular Bruker EPR cavity (ER4102T) which was equipped with an Oxford helium (ESR 900) cryostat. The microwave frequency was measured using a Systron Donner (6054D) frequency counter, and the magnetic field was measured using a Bruker gaussmeter (ER035M). The measured *g* values were corrected for an offset against a known *g* standard (DPPH).

The X-band EPR powder spectra have been analyzed using a self-written simulation and fit program which is based on the work of Rieger (*30*) using a modified Levenberg−Marquardt nonlinear least-squares fit algorithm (*31*). This simulation routine is based on second-order perturbation theory and can deal with an arbitrary number of nuclei with noncollinear **g** and **hf** tensors.

RESULTS

Several paramagnetic states can be observed when EPR spectroscopy is used to investigate cytochrome *c* oxidase at low temperatures. EPR signals have been detected from Cu_A, heme *a*, and Mn(II) (*32*). These signals are shown in Figure 1. In Figure 1A, the EPR signal from Cu_A is obscured by that resulting from Mn(II) while that of heme *a* is clearly observed. This Mn(II) EPR signal can however be suppressed by growing the bacteria in a Mn-depleted medium as shown

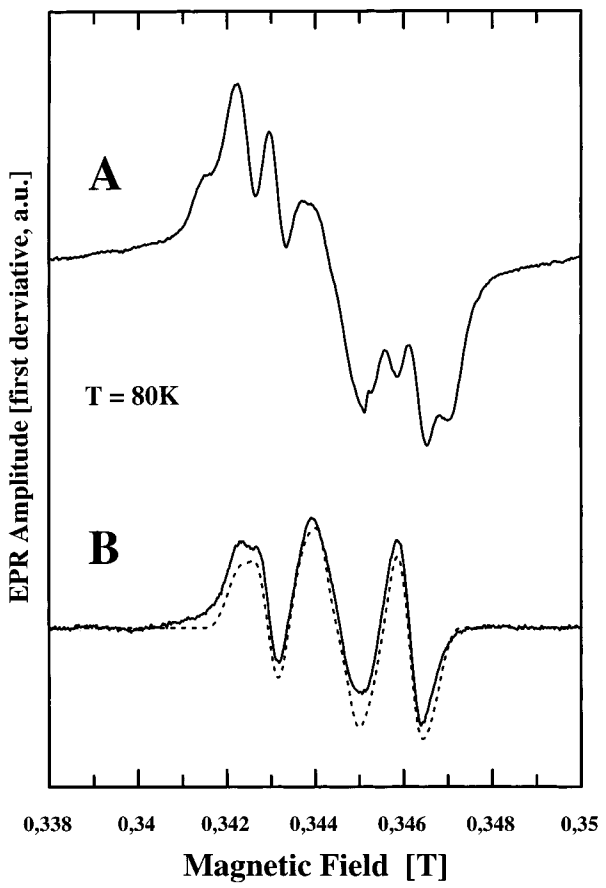

FIGURE 2: EPR spectra of the tyrosine radical observed after treatment of COX with H_2O_2 recorded at 80 K. Both spectra were obtained by subtraction of the spectrum of untreated COX at the same pH and temperature from that of the peroxide-treated enzyme: (A) COX with unlabeled tyrosine and (B) COX with tyrosine-d_4. Solid lines represent the experimental values; the dotted line represents the best simulation (fit). The obtained **g** and **hf** tensors are given in Table 1. The experimental conditions were as follows: microwave power, 0.1 mW; field modulation, 12.5 kHz; and amplitude, ±0.2 mT peak to peak.

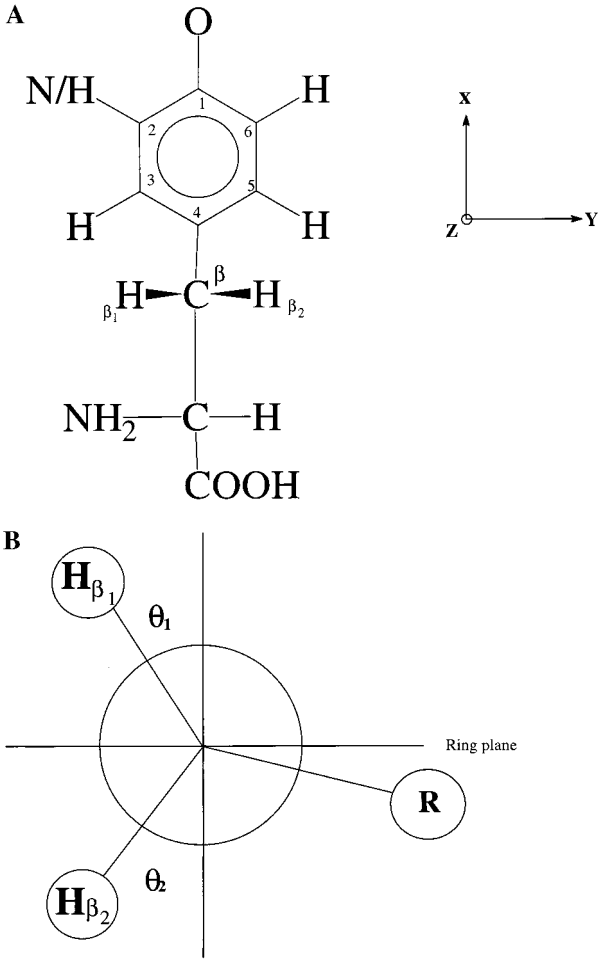

FIGURE 3: (A) Molecular structure, numbering scheme (inner numbers), and axes system of the tyrosine radical. (B) Conformation of β-methylene protons $H_{\beta 1}$ and $H_{\beta 2}$ and the corresponding dihedral angles θ.

in Figure 1B. COX from *P. denitrificans* grown in the absence of Mn is fully active, and the EPR spectrum exhibits no Mn(II) EPR signal at X-band frequencies. Here the EPR signal from Cu_A is clearly resolved.

In the presence of 1 molar equiv of H_2O_2, an additional EPR signal is observed at a g value of ~2 typical of organic radicals (asterisk in Figure 1C). Samples containing Mn(II) which are treated with H_2O_2 also provide evidence of the presence of this radical signal, although it is more difficult to observe (data not shown). COX which was pretreated with cyanide did not exhibit a radical EPR signal after addition of H_2O_2, indicating that an active intact binuclear center site is needed for formation of the radical.

Figure 2A is a closeup of the $g \sim 2$ region of spectra recorded at 80 K. To assist in the analysis of the spectra, difference spectra taken by subtraction of the EPR signal arising from the Cu_A center in the untreated COX preparation are shown (Figure 1B). It can be clearly seen that the observed EPR signal reveals a partially resolved structure which is most probably due to hyperfine interactions. The signal is centered at an average g value slightly shifted downfield from ~2 which is a value typical for organic radicals such as semiquinone anions (*33*) or of neutral tyrosine radicals [e.g., photosystem II (*34*) or ribonucleotide reductase (*35*)]. Using DPPH as a **g** marker, this value has been determined to be ~2.0055 (±0.0005). The error margin here is quite large due to the fact that the signal is superimposed on that of the Cu_A, making an exact determination difficult.

At X-band frequencies (9 GHz), the overall width of an EPR spectrum can be dominated either by unresolved hyperfine (hf) interactions, by **g** anisotropy, or by a combination of both. At present, several tyrosine radicals have been observed in biological systems, and it has been shown that their hyperfine structures are very sensitive especially to the orientation of the ring headgroup relative to the protein backbone (*36*, *37*). More specifically, the dominant features in the spectra arise from distinct hyperfine couplings (hfcs) of the β-methylene protons (β_1 and β_2 at C_β which forms a bond with the tyrosine ring at position C_4; see Figure 3A for labeling), while those of the α-protons at the phenol ring (C_3, C_5, C_2, and C_6) are smaller in magnitude, more anisotropic and almost invariant.

The use of cw-EPR spectroscopy in quantifying relative amounts of different signals in the same sample can be misleading, especially in biological samples where different species may exhibit different saturation properties, for example, due to the presence of fast relaxing species such as transition metal ions close by. To avoid such problems,

Table 1: Experimental **g** Tensor (g_{ii}) and Hyperfine Tensor (A_{ii}[mT]) Principal Values for the Observed Tyrosine Radical in Cytochrome *c* Oxidase of *P. denitrificans*

radical	tensor element	g_{ii}[b]	A_{ii} (β_1)	A_{ii} (β_2)
Tyr•[a]	*xx*	2.0099 (5)	1.62 (2)	1.48 (2)
	yy	2.0064 (5)	1.78 (2)	1.39 (2)
	zz	2.0020 (5)	1.37 (2)	1.23 (2)
	$^1/_3$ Tr	2.0061 (5)	1.59 (2)	1.37 (2)

[a] From fits of the EPR powder spectra (Figure 2A). Values in parentheses are estimated errors in the last digit. [b] Values were calibrated against a DPPH sample ($g = 2.00351$).

this signal has been quantified using a pulsed-EPR technique. This method is not restricted by relaxation effects as long as the microwave pulse repetition time is longer than the longitudinal relaxation time T_1 ($3T_1$) and the pulse separation time (τ) is much smaller than the transverse relaxation time T_2. A two-pulse echo sequence ($\pi/2 - \tau - \pi$) is applied, and the amplitude of the resulting echo is detected while sweeping the magnetic field, resulting in a field-swept EPR spectrum. The resulting spectrum was simulated using the known EPR parameters for the signals arising from Cu_A and heme *a* and including typical EPR parameters determined from this radical ($g_{av} \sim 2.0055$ and a line width of 30 G). With the assumption that the yield of the EPR signal arising from Cu_A is one per COX complex, the amount of free radical was estimated to be ca. $20 \pm 5\%$. This experiment has been performed with several different preparations, making it unlikely that this is a preparation-dependent artifact.

To identify the origin of this species, a selective labeling experiment was performed. Ring-deuterated ($^2H_4$) tyrosine was incorporated into the protein (see Materials and Methods). The two β-methylene protons (at position C_β) remain ^{1}H-protonated. The EPR signal was generated in the same way and is shown in Figure 2B. A very distinct change has occurred to the resolved structure of the spectrum. The hyperfine couplings arising from the ring α-protons are now no longer resolved. Line shape analysis indicates that the remaining three lines result from nearly isotropic hfcs, and the hyperfine lines leading to this prominent three-line structure have no observable anisotropy. A small degree of hyperfine anisotropy is quite typical for hfcs of such β-protons as has been seen previously by electron nuclear double-resonance (ENDOR) spectroscopy of tyrosine radicals in photosystem II and ribonucleotide reductase (*36*, *37*). Thus, these three lines with an intensity pattern of 1:2:1 (as seen from integrating the spectrum, data not shown) are expected from the two β-protons when the hfcs of β_1 and β_2 are equal (or very similar). However, when known β-proton hfcs for tyrosine radicals and their known and/or measured anisotropies are taken from the literature, the observed line shape can still only be fitted by also assuming a certain degree of **g** anisotropy [$\Delta g(g_{xx} - g_{zz}) \sim 0.007-0.008$, a value typical of tyrosine radicals (*34*, *35*, *38*)]. The actual values used in the simulation are contained in Table 1, although a more accurate determination of the exact **g** tensor would require performing EPR experiments at much higher microwave frequencies and magnetic field strengths. Such experiments are currently underway in our laboratory.

The fit of the spectrum (Figure 2B, dashed line) based on two nearly isotropic proton hfcs and a **g** tensor is in good agreement with the experimental spectrum. This holds in particular for the peak positions and the zero crossings. The hyperfine and **g** tensor principal values obtained from the fit of Figure 2B are given in Table 1.

The isotropic coupling [$A_{iso} = {}^1/_3(A_{xx} + A_{yy} + A_{zz})$] for the β-methylene protons can be calculated from the tensor components in Table 1. The isotropic hfc of a β-proton is related to the neighboring carbon spin density $\rho^\pi(C_4)$ by the relationship

$$A_{iso}(H_\beta) \sim \rho^\pi(C_4)[B' + B'' \cos^2 \theta] \quad (1)$$

where B' and B'' are empirical constants, $\rho^\pi(C_4)$ is the spin density distribution on C_4, and θ is the dihedral angle between the α-carbon p_z axis and the projected $C_\beta-H_\beta$ bond (*39*). If a value of 5.8 mT for B'' (B' is negligible) is assumed as has been suggested for tyrosine radicals (*40*) and a spin density $\rho^\pi(C_4)$ of 0.38 is assumed (*37*), the resulting dihedral angles θ_1 and θ_2 would be 38 and 32°, respectively. If sp^3 hybridization is assumed, then the resulting dihedral angle of the adjacent C_α atom would be much closer to the ring plane than to the ring normal.

These labeling results together with the fact that the parameters from other well-characterized protein-based radicals such as tryptophan and glycyl radicals, observed in pyruvate formate lyase (*41*) or ribonucleotide reductase (*42*), are not suitable for simulating the EPR spectrum suggest that the observed radical originates from a tyrosine residue.

The EPR spectrum from the fully protonated tyrosine (Figure 2A) also shows additional splitting due to the ring α-protons. For the simulations and fits of this spectrum, the parameters obtained from the deuterated species should be held constant. An unequivocal assignment of the tensor components is at present not possible; further experiments (e.g., ENDOR) are necessary to aid the assignment, which will then indicate whether the observed hyperfine structure arises from four ring α-protons or three protons and one nitrogen atom. Such experiments are currently being performed in our laboratories.

DISCUSSION

Results presented here provide direct evidence for a radical species generated in the reaction of COX with H_2O_2. Using residue specific isotope labeling, the radical signal observed in the EPR spectrum could clearly be assigned to a tyrosine residue.

As mentioned above, such a radical species has been proposed for the so-called $\mathbf{P_m}$ intermediate where the binuclear center is at the two-electron reduced level and the oxygen−oxygen bond is already broken. This $\mathbf{P_m}$ state can be generated by addition of substoichiometric or stoichiometric amounts of hydrogen peroxide to the oxidized enzyme (*11*) and is characterized by an absorbance band at 607 nm. [This treatment results in a mixture of **O**, **P**, **F**, and **F′** states (whereby the yield of **P** is low at low pH), as not all the H_2O_2 will react with the enzyme especially within the short incubation time before freezing. **F** would be generated by a reaction of two H_2O_2 molecules with one COX, increasing the percentage of unreacted COX (**O**). Thus, the yield of 20% observed for the radical is reasonable as only the **F′** state is expected to yield the radical signal.] At pH 6, however, a species with an absorbance band at 580 nm

[CCO-580 (*11*)] is formed, which has been termed **F′** (*43*) as it has an optical spectrum similar to that of compound **F**. However, as we find that **F′** can be generated directly from the 607 nm species just by lowering the pH and without further input of electrons (to be published elsewhere), it is clear that the two species share the same electronic structure of the binuclear center. The change in the absorbance spectrum may be explained with different local environments due to Cu_B ligands or nearby protein sites being differently protonated in the two species.

This has been noted by Michel (*26*) who has proposed an oxoferryl−cupric species with an additional protein radical as structures for both $\mathbf{P_m}$ and **F′** with the difference between the two states being a hydroxide ion bound to the Cu_B in $\mathbf{P_m}$ that is protonated and thus a water in **F′**. Therefore, as the hydroxide ion could, through antiferromagnetic coupling, make the tyrosine radical EPR-undetectable, we have performed the experiment at pH 6 rather than at pH 8.

It should be mentioned here that Weng and Baker (*10*) proposed that **P** is an oxoferryl species with a hydroxylated Cu_B. But in analogy to cytochrome *c* peroxidase, they proposed a tryptophan to be the radical species. Radical species have also previously been observed in the reaction of COX with H_2O_2 (*11*) and during the turnover reaction of COX with cytochrome *c* (*44*); however, in both cases, the identity of the observed radicals was not resolved. Here it has been clearly shown that a tyrosine is the radical species. In the study by Fabian and Palmer (*11*), a radical species was observed with a line width similar to that observed here. There are slight experimental differences which may explain the observed differences in the EPR signal. Here a stoichiometric amount of H_2O_2 has been used as opposed to an excess in ref *11*. Probably more important is the fact that the unresolved spectrum in ref *11* was recorded at 12 K and at microwave powers which are already saturating. The signal observed here reveals hyperfine resolution at 80 K. At temperatures below 60 K, this resolution is lost due to an additional interaction (probably a dipolar interaction with a nearby paramagnetic center), resulting in the loss of the hyperfine resolution yielding a signal similar to that observed in ref *11*. This loss of resolution is a further indication that the observed radical is in the immediate vicinity of the binuclear center. Further pulsed-EPR studies are currently in progress to measure this distance accurately.

Very recently, Chen et al. (*45*) reported a spin trap study of bovine mitochondrial COX treated with an excess of H_2O_2 at pH 7.4. Under conditions where COX was inhibited with *N*-ethylmaleimide (which alkylates free thiols on the surface of the enzyme), a nitroso spin trap indicated the presence of a protein-derived radical. Both tryptophan and tyrosine were postulated as possible candidates for the origin of this radical adduct. Comparison with a model tyrosine adduct using a similar technique led to the suggestion that the origin of the radical adduct was indeed a tyrosine. Whereas the direct comparison of these spin trap results with other well-known, protein-bound tyrosine or tryptophan radicals is not easy, the result of this indirect detection and assignment would be in agreement with the results presented here for native COX.

The most likely candidate appears to be Tyr 280 that is covalently linked to the Cu_B ligand His 276 and close enough to the binuclear center to participate in the O_2 reduction process. Mutation of this residue to either phenylalanine or histidine yields an essentially inactive enzyme (*46*, *47*) with incomplete incorporation of Cu_B. The covalent linkage to His 276 might optimize the geometry and modulate the redox potential and p*K* of Tyr 280, thus favoring hydrogen abstraction by oxygen bound to the binuclear center although the cross-link itself is most likely formed by a side reaction of the tyrosine radical formed prior to the establishment of the cross-link during the first turnover of the enzyme.

Further characterization of the Tyr 280-derived EPR signal in terms of its pH sensitivity, its dependence on the H_2O_2 concentration, and its coupling to other sites is currently under way in our laboratories.

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