Insights into the Functional Role of the Tyrosine−Histidine Linkage in Cytochrome *c* Oxidase

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Received October 21, 1999

Recent crystallographic studies of cytochrome *c* oxidase (CcO) have revealed a unique and unexpected posttranslational modification in the enzyme active site. This modification results in a tyrosine cross-linked at C6 to the ϵ-nitrogen of a histidine residue (Figure 1).[1] This finding has raised the question of the role of this novel cross-link in enzyme catalysis. We report here the first experimental studies to address this issue. Cytochrome *c* oxidase catalyzes the four-electron reduction of molecular oxygen to water, an essential step in the respiratory chain.[2] The active site of the enzyme contains a binuclear center composed of heme a_3 and Cu_B (Figure 1). Since its discovery, a number of critical functional roles have been offered for the unique cross-linked residue. In most models, the tyrosine, which is located at the end of a possible proton channel to the surface (K-channel), provides a hydrogen to the distal oxygen of a dioxygen species bound to the heme. The detailed mechanism of this process (proton or hydrogen atom transfer) differs in the various proposals. In their seminal work on the structure of the enzyme from bovine heart mitochondria, Yoshikawa and co-workers proposed[1a] proton transfer from Tyr244 to a ferric peroxide to generate a hydroperoxo adduct. In a subsequent step, Cu_B^{1+} was suggested to provide an electron via the His^{240}-Tyr^{244} cross-link to cleave the O−O bond of the ferric hydroperoxide. On the other hand, several groups have speculated that the cross-linked tyrosine might serve as a hydrogen atom donor during the reduction of O_2.[1b,2b,c,3] In this model, the metals in the active site provide three of the four electrons required for O−O bond cleavage by oxidation of the Fe_{3a}^{2+}/Cu_B^{+} binuclear center to oxoferryl (Fe^{4+}═O) and HO−Cu_B^{2+}. On the basis of the proximity of the cross-linked tyrosine, it was suggested that this residue might supply both the proton and the fourth electron required for this transformation, via hydrogen atom transfer from the neutral tyrosine. Thus, a tyrosyl radical would be generated, placing CcO in the company of a growing number of proteins that utilize redox active amino acids during catalysis.[4] The feasibility of these functional roles, as well as others,[5] critically depends on the perturbation of the physicochemical properties of the tyrosine as a result of the covalent bond to histidine. We provide the first experimental insight into these issues.

To evaluate the modulation induced by the cross-link, we have prepared 2-imidazol-1-yl-4-methylphenol (**1**).[6] The UV−vis spectrum of **1** showed a λ_{max} at 286 nm at pH 3−7, and a λ_{max} at 304 nm in basic solution (pH >10). Spectrophotometric titration of **1** at 314 nm revealed a pK_a of 8.60 ± 0.04 for the phenol moiety (Figure 2).[7] Careful examination of the spectra at 230 nm revealed a second pK_a at 5.54 ± 0.12, assigned to the imidazole group of **1** (inset Figure 2). Titration of *p*-cresol under identical conditions produced a pK_a of 10.23 ± 0.09. Thus, the covalently linked imidazole group perturbs the acid dissociation constant of the phenol by more than 1.5 orders of magnitude. This modulation is most likely due to stabilization of the phenolate through an inductive effect that may be even more pronounced in the protein by coordination of the imidazole to Cu_B.[8]

Electrochemical experiments were performed to assess the influence of the cross-link on the redox properties of **1**. Cyclic voltammetry at pH 11.5 produced irreversible waves for both **1** and *p*-cresol, with the anodic peak potential of the former 66 ± 3 mV more positive.[9] This increase of the peak potential is

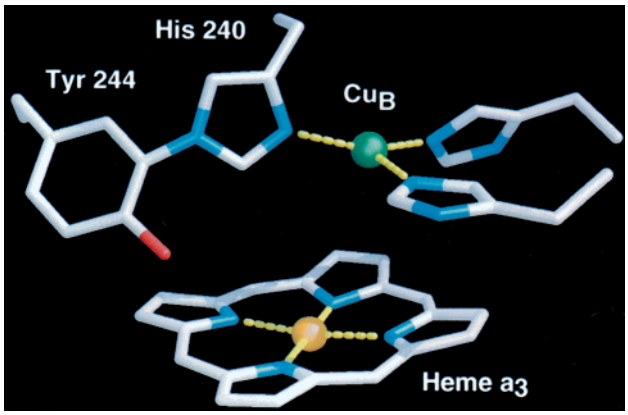

Figure 1. Binuclear center of cytochrome *c* oxidase from bovine heart in the reduced state.[1a] Coordinates 10CR from RCSB PDB. The axial histidine ligand to the heme and the heme side chains are omitted for clarity.

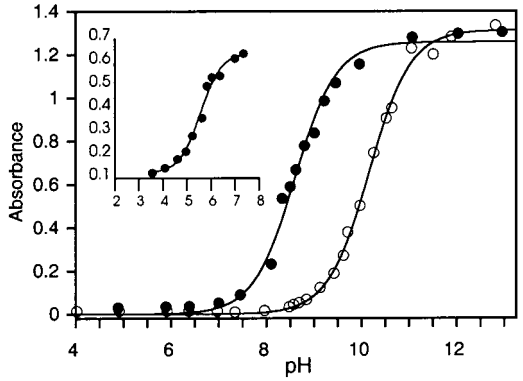

Figure 2. Spectrophotometric titrations of aqueous solutions of **1** (0.4 mM, closed circles) and *p*-cresol (0.56 mM, open circles). The titration was monitored at 314 nm for **1** at which wavelength only the deprotonated form absorbs. Inset shows pH dependence of the absorbance at 230 nm. For *p*-cresol the titration was monitored at 297 nm.

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(1) (a) Yoshikawa, S.; Shinzawaitoh, K.; Nakashima, R.; Yaono, R.; Yamashita, E.; Inoue, N.; Yao, M.; Fei, M. J.; Libeu, C. P.; Mizushima, T.; Yamaguchi, H.; Tomizaki, T.; Tsukihara, T. *Science* **1998**, *280*, 1723−1729. (b) Ostermeier, C.; Harrenga, A.; Ermler, U.; Michel, H. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 10547−10553. (c) Buse, G.; Soulimane, T.; Dewor, M.; Meyer, H. E.; Bluggel, M. *Protein Sci.* **1999**, *8*, 985−990.

(2) (a) Ferguson-Miller, S.; Babcock, G. T. *Chem. Rev.* **1996**, *96*, 2889−2907. (b) Gennis, R. B. *Biochim. Biophys. Acta* **1998**, *1365*, 241−248. (c) Michel, H. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 12819−12824. (d) Michel, H. *Biochemistry* **1999**, *38*, 15129−15140.

(3) Proshlyakov, D. A.; Pressler, M. A.; Babcock, G. T. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 8020−8025.

(4) Stubbe, J.; van der Donk, W. A. *Chem. Rev.* **1998**, *98*, 705−762.

(5) In addition to involvement in O−O bond cleavage, the Tyr-His cross-link may serve other roles. Yoshikawa[1a] suggested that the cross-linked tyrosine is deprotonated in the fully oxidized state of the enzyme. Thus, protonation of Tyr244 is required to prime the enzyme for catalysis.[1a] Indeed, in the reductive half of the catalytic cycle, the fully oxidized enzyme has been reported to take up two protons as well as two electrons to reduce the binuclear site to its Fe^{2+}/Cu^{+} oxidation state (R-state) (reviewed in ref 2b). It has been proposed that these protons are delivered to the binuclear center via Tyr244, and that one of the protons is used to protonate Tyr244 and prepare it for its role in O−O cleavage.[2b] An alternative view of proton movement is presented in refs 2c,d.

(6) Full experimental details can be found in the Supporting Information. See also: Strehlke, P. *Eur. J. Med. Chem.* **1979**, *14*, 227−230.

(7) The pK_a values were determined by two independent titrations of 0.4 mM analyte using 17 different buffer solutions adjusted to 0.3 M ionic strength with KCl.

10.1021/ja993774s CCC: $19.00
Published on Web 02/26/2000

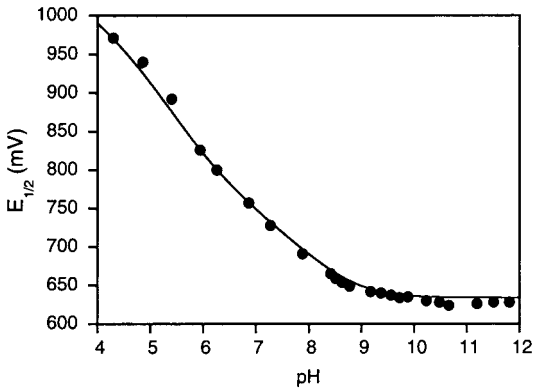
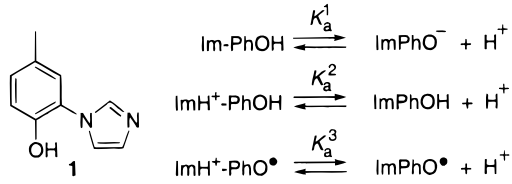


Figure 3. pH dependence of the midpoint potential of **1** in aqueous buffer determined by DPV. Potential values are versus NHE. Working electrode, glassy carbon; counter electrode, Pt wire; reference electrode, $Ag^+/AgCl$. All buffers were adjusted to identical ionic strengths using KNO_3 electrolyte. Solid line produced by fitting the data to the following equation:[18] $E_m = \text{Cst} + 0.059 \log\{[H^+]^2 + K_a^1[H^+] + K_a^1K_a^2\} - 0.059 \log\{[H^+] + K_a^3\}$.

Scheme 1



consistent with the imidazole withdrawing electron density from the phenolate. The pH dependence of the midpoint potential for one-electron oxidation of **1** determined by differential pulse voltammetry (DPV) is shown in Figure 3. The data are best fit as a one-electron oxidation process that is affected by three different dissociation constants.[10] Two of the three pK_a values thus obtained ($pK_a^2 = 5.7 \pm 0.20$, and $pK_a^1 = 8.90 \pm 0.08$) agree reasonably well with those determined by spectrophotometric titration of **1** (Scheme 1).[11] On the basis of the direction of the change in slope at the third pK_a ($pK_a^3 = 4.80 \pm 0.24$), it is associated with protonation of the imidazole of the oxidized species. The alternative, protonation on oxygen of the phenol radical, would require significantly lower pH values.[12]

Combining the experimentally determined values of the equilibrium acidities (pK_a) and oxidation potentials of their conjugate anions ($E_{1/2}$ at pH 11.5) for **1** and *p*-cresol allows an estimation of the influence of the cross-link on the bond dissociation energy of the phenol oxygen−hydrogen bond using a thermodynamic cycle.[13] The increase in the oxidation potential, coupled with the depression of the pK_a, leads to a 0.7 kcal/mol decrease in BDE energy of the O−H bond in **1** compared to *p*-cresol.[13] Thus, this study supports previous proposals[5] that the cross-link will facilitate proton delivery to the binuclear center in the reductive half reaction by lowering the tyrosine pK_a. In the oxidative half reaction, the hydrogen atom transfer model requires that hydrogen atom abstraction is facilitated by the cross-link without stabilizing the resulting His-Tyr radical so much that its subsequent one-electron reduction becomes less favorable. Compound **1**, with its lowered pK_a and slightly increased oxidation potential, provides such a delicately balanced system.[14]

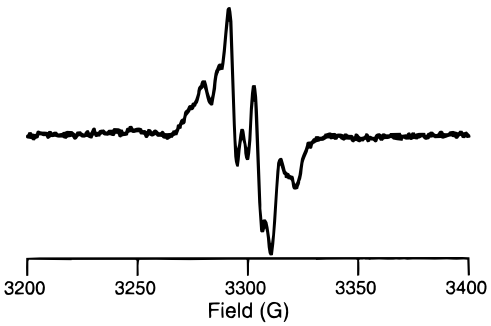


Figure 4. X-band EPR spectrum of the radical form of **1**. Instrument settings: frequency 9.27 GHz, power 100 μW, modulation amplitude 1 G, time constant 0.032 s, and temperature 77 K.

Very recently, MacMillan and co-workers reported the EPR signal of a radical generated in CcO.[15] The signal displayed a g_{iso} value of ~2.0055 with a spectral width of about 80 G, and was proposed to originate from the cross-linked tyrosine.[16] While the functional importance of this signal remains to be established, its assignment by double resonance techniques will be assisted by the availability of spectroscopic data on the radical form of **1**. In a preliminary experiment, we have generated the latter by UV-photolysis of a frozen solution at pH 10 resulting in the EPR signal shown in Figure 4. The overall width and g_{iso} of the signal (2.0058) are similar to those reported for the enzyme.[17] High-frequency EPR and double resonance techniques on the enzyme signal and **1**$^\bullet$ should enable the assignment of the hyperfine interactions and establish whether the former is indeed associated with the cross-linked tyrosine residue. Such experiments as well as incorporating **1** into a copper-containing model are currently underway.

Acknowledgment. This work was supported by the Department of Chemistry and the Snyder Scholar Fund at the University of Illinois. We are grateful to Profs. Gennis and Martinez (UIUC) and Prof. Babcock (Michigan State) for sharing unpublished data and for stimulating discussions. We thank Prof. Suslick (UIUC) for use of electrochemical equipment and Prof. Gerlt for help with preparing Figure 1.

Supporting Information Available: Synthetic procedures for the preparation of **1** and fits of the DPV data to alternative models (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(8) An *N*-linked imidazole is not expected to lower the pK_a of the phenol by resonance stabilization of the conjugate anion. The increased acidity of the phenol is therefore best explained as an inductive electron-withdrawing effect. The influence of copper coordination will depend on its oxidation state as well as coordination geometry. Generally, coordination will increase the inductive electron-withdrawing effect, but dπ−pπ back-bonding may offset this. Note also that the cross-link lowers the basicity of the imidazole nitrogen, which will weaken its coordinating ability.

(9) This value was derived from five independent scans at 100 mV s^{-1} and probably represents an upper value. At 10 mV s^{-1}, **1** showed a peak potential 73.0 ± 4.9 mV more positive than *p*-cresol.

(10) Two independent determinations of the pH dependence of the half-wave potential both showed a significantly better fit to three than to two dissociation constants. Figures showing alternative fits are provided in the Supporting Information.

(11) While the pK_a values determined from two independent electrochemical experiments proved reproducible within 0.2, the spectrophotometric values are more accurate since the former are extracted from an irreversible redox system, which precludes accurate determination of $E°$.

(12) Dixon, W. T.; Murphy, D. *J. Chem. Soc., Faraday Trans. 2* **1976**, *72*, 1221−1230.

(13) This procedure has been used widely by Bordwell and co-workers and provides the following equation: $\Delta\text{BDE(ArO−H)} = F\Delta E^0_{1/2}(\text{ArO}^-) + 2.302RT\Delta pK^1(\text{ArOH})$. Bordwell, F. G.; Bausch, M. J. *J. Am. Chem. Soc.* **1986**, *108*, 1979−1985. For a rigorous application reversible redox potentials are required. Despite this Bordwell[13b] and Parker[13c] have suggested that the error introduced by using irreversible potentials for a family of compounds is not significant or is fortuitously canceled. (b) Bordwell, F. G.; Cheng, J.-P.; Ji, G.-Z.; Satish, A. V.; Zhang, X. *J. Am. Chem. Soc.* **1991**, *113*, 9790−9795. (c) Parker, V. D. *J. Am. Chem. Soc.* **1992**, *114*, 7458−7462 and references therein; correction: Parker, V. D. *J. Am. Chem. Soc.* **1993**, *115*, 1201.

(14) An alternative interpretation is that the relatively minor perturbation in the BDE indicates that the cross-link merely serves a structural role. The importance of the cross-link for proper assembly of the binuclear center has been shown by site-directed mutagenesis: Das, T. K.; Pecoraro, C.; Tomson, F. L.; Gennis, R. B.; Rousseau, D. L. *Biochemistry* **1998**, *37*, 7, 14471−14476.

(15) MacMillan, F.; Kannt, A.; Behr, J.; Prisner, T.; Michel, H. *Biochemistry* **1999**, *38*, 9179−9184.

(16) If the signal is indeed associated with the cross-linked tyrosine one might have expected significant broadening of the signal or even elimination of any signal due to magnetic coupling between the radical and Cu_B^{2+} via the direct covalent connection (Figure 1).

(17) The two signals differ in line shape since the signal in CcO is largely governed by the orientation of the two β-protons of the Tyr residue, while the line shape for **1**$^\bullet$ is dominated by three isotropic methyl protons (1:3:3:1 pattern with A_{iso} of 10−11 G).

(18) Clark, W. M. *Oxidation−Reduction Potentials of Organic Systems*; Krieger Publishing Co.: Huntington, NY, 1972.