UV optical absorption by protein radicals in cytochrome c oxidase

Denis A. Proshlyakov*

Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824, USA

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Abstract

The UV properties of key oxygen intermediates of cytochrome c oxidase have been investigated by transient absorption spectroscopy. The temporal behavior of \( P_m \) species upon aerobic incubation with CO or in the reaction with \( \text{H}_2\text{O}_2 \) is closely concurred by a new optical shift at 290/260 nm. In the acid-induced conversion of \( P_m \) to \( F \), it is replaced by another shift at 323/288 nm. The wavelength and intensity of the UV signal observed in \( F \) match closely the properties of model \( \text{Trp} \) in agreement with results of ENDOR studies on this species. The UV spectrum of \( \text{Tyr} \) gives the closest match with the 290/260 nm signal observed in \( P_m \). On the basis of analysis of possible UV chromophores in \( \text{CcO} \) and similarity to \( \text{Tyr} \), the 290/260 nm signal is proposed to originate from the \( \text{H}_2\text{Cu}-\text{Y}244 \) site. Possible effects of local environment on UV properties of this site are discussed.

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1. Introduction

The question as to how \( \text{O}_2 \) is reduced by the terminal enzyme of the mitochondrial respiratory chain, cytochrome \( \text{c} \) oxidase (CcO), has been a controversial issue over the past decade. While there has long been consensus about structures of most reactive intermediates \([1,2]\), detailed structure of a \( P_m \) species remains an open question. Oxidation state of \( P_m \) corresponds to that of peroxide in the solution (hence “peroxy” or \( \text{P} \)), since it is produced when two electrons and \( \text{O}_2 \) react (either sequentially or as \( \text{H}_2\text{O}_2 \) with the oxidized enzyme \([3–7]\). Resonance Raman studies on \( P_m \) and successive \( F \) (or “ferryl”) species showed that the O–O bond in \( P_m \) is already broken \([5,8,9]\) and that this occurs immediately following transfer of the second electron to the primary \( \text{Fe}^{II}-\text{O}_2 \) species \([7]\). Release of one of the oxygen atoms from the active site, in the form of water, strongly supported this assignment \([10]\). Because metal centers in the binuclear center could supply only three \( e^- \) out of four \( e^- \) required for cleavage of the O–O bond, the existence of an additional redox center has been proposed \([4,5,7,9,11]\).

In the absence of spectroscopic evidence for involvement of an additional electron donor, discovery of an unusual cross-link between \( \text{H}^{240} \) and \( \text{Y}^{244} \) (bovine nomenclature) hinted its identity \([12–15]\). By utilizing a selective reactivity of iodide with aromatic radicals, it became possible to show that the active site of \( P_m \) does, indeed, contain an extra oxidizing equivalent \([16]\). Amino acid sequencing of the labeled protein pinpointed its location to the \( \text{H}_2\text{Cu} \)-\( \text{Y}244 \) dimer.

Spectroscopic detection of \( \text{Y}^{244} \) remains elusive. A close proximity of putative radical site to nearby paramagnetic \( \text{Cu}_8 \), enhanced by the presence of covalent bridging, makes both centers invisible for traditional EPR spectroscopy \([4,7,17]\). While optical absorption by radicals \([18–22]\) can be used for their detection in non-heme enzymes \([23–26]\), intense and variable visible absorption by the heme \([27]\) obscures this region in CcO.

Because of the critical role the \( \text{H}^{240} \)-\( \text{Y}^{244} \) site is expected to play in catalysis, a number of studies have
been initiated to detect and characterize this site in CcO. Particular emphasis is placed on vibrational characterization of model compounds in either UV [28,29] or IR regions [30–32], as reviewed elsewhere in this issue. An earlier study suggested that formation of $P_m$ can cause conformational changes detectable in the UV region and suggested involvement of Tyr and Trp residues [33]. This study explores UV optical properties of three semi-stable reactive intermediates—$P_m$, $F^*$, and $F$—in a view of current knowledge about mechanisms of CcO catalysis.

2. Materials and methods

Beef heart CcO was purified, as described earlier [16]. Room-temperature optical absorption measurements were carried out using a Hewlett Packard spectrophotometer model 8453 (Agilent Technologies, USA). For the peroxide reaction, a small (~1/1000) volume of $H_2O_2$ stock was injected into sample of oxidized CcO, and optical changes were followed between 0.1 s and 10 min. $P_m$ was generated by aerobic incubation of 2–5 μM enzyme with CO delivered either by bubbling for 1 min or injection of CO-saturated buffer. $F^*$ species was generated from $P_m$ upon acid jump, as follows: $P_m$ was generated by mixing of ~150 μM CcO sample at pH 8.5 with CO-saturated buffer without gas phase, using two microsyringes. High concentration of reactants and sub-stochiometric amount of CO ensured that formation of $P_m$ proceeded rapidly until CO was exhausted. CO removal was completed by aerobic incubation of 20–30 μl of sample for several minutes, while stirred. Absence of CO was imperative for high yield of $F^*$ in the next step. A concentrated sample of $P_m$, thus generated, was injected into large excess of buffer at pH 6.5 and rapidly mixed. To

Fig. 1. Formation of $P_m$ in aerobic reaction of oxidized CcO with CO. Panel A: UV–Vis difference absorption spectra observed at 48, 78, 110, 156, 231, and 333 s following bubbling of 5 μM CcO with CO for 60 s at pH 8.5. Panel B: Kinetics of optical changes observed at 607 nm (trace a) and difference $Δε_{291} – Δε_{258}$ (trace b) under conditions described for panel A. Traces c and d, respectively, were observed when CO-saturated buffer ([CO]$_{final}$=70 μM) was added instead of bubbling.
account for spectral changes occurring primarily due to sample dilution and bulk changes upon lowering of pH, a parallel experiment was repeated using Ar instead of CO.

3. Results

3.1. Pm formation by CO+O2

Fig. 1A shows optical changes observed upon incubation of oxidized CcO in the presence of both CO and O2 [34]. Formation of a low-spin ferryl-loxo heme causes red shift of the Soret band that overwhelms the blue region. An increasing intensity of $\alpha$-band at 607 nm is a characteristic signature of Pm formation. A new difference pattern is prominently seen in the UV region as a shift of a band from ~260 nm in resting enzyme to 290 nm in Pm. Half-width of negative and positive bands are 14 and 19 nm, respectively. A shoulder at ~304 nm and a weak band at ~336 nm can be seen in Pm. Isosbestic points at 249, 270, and 341 nm indicate that a single process contributes to observed UV changes, and no side reactions occur on the time scale of minutes.

To examine if a 290/260 nm band arises from Pm or a separate species, temporal changes at 607 and 290/260 nm are compared in Fig. 1B. It was found that the

Fig. 2. Acid-induced optical changes in Pm and Ox. Panel A: Optical changes observed for Pm (a) and oxidized (Ar-treated) CcO (b) at 5 min following pH jump from 8.5 to 6.5. Panel B: Trace a, spectrum of Pm→F$^*$ transition obtained as a difference of traces a and b in panel A; trace b, difference spectrum of F$^*$ versus oxidized CcO obtained by subtracting contribution of Pm from trace a, as described in text.
behavior of the 290/260 band over the first ~20 min at room temperature does, indeed, concur with that of the 607-nm band. This was also the case when the rate of Pm formation was varied due to concentration of CO in the solution. Additional process begins to contribute in the UV region at later times and most likely is associated with stability of the enzyme, since it did not depend on initial kinetics.

3.2. Peroxide reaction

UV optical properties of Pm and F in the peroxide reaction were reexamined in time-resolved measurements using concentrations of H2O2 of 0.5 to 10 mM, i.e. × 100 higher than that in the previous study [33]. Optical changes around 280 nm were identical to those observed in the CO + O2 reaction and closely followed evolution of Pm (not shown). No lag was detected between development of Pm and the UV shift, even at highest concentrations of H2O2. This contradicts the previous assignment of the 280-nm shift to conformational changes at CuB site prior to Pm formation [33], as such two phases would be better resolved at high reaction rates. No UV signals were detected in F, except for gradual background changes, presumably due to structural damage.

3.3. pH-induced Pm–F* transition

Fig. 2A compares spectral changes in the oxidized enzyme and Pm over the first 5 min following change of pH from 8.5 to 6.5. Conversion of Pm into F* is evident from loss of intensity at 607 nm with concurrent increase at 580 and 530 nm (trace a) [35]. Comparison between CO- (trace a) and Ar-treated (trace b) samples shows that the Pm–F* transition involves significant changes in the UV region, in addition to background changes observable with Ox. Trace a in Fig. 2B shows difference between traces a and b in Fig. 2A and corresponds to isolated contribution of Pm–F* transition. This double difference spectrum can be presented as (Facid−OXacid) − (Pm base − Oxbase), where the latter term corresponds to formation of Pm at high pH. Hence, difference spectrum of F* formation at acidic pH can be isolated by subtracting spectrum of Ox−Pm transition (Fig. 1A), as shown by trace b. To account for partial completion of Pm–F* transition and decay of both species, spectrum of Pm from Fig. 1 was reduced upon subtraction so that the resulting spectrum did not exhibit significant 607-nm peak. The red region of trace b (Fig. 2B) closely matches experimental spectrum of F/F* formed by H2O2 at acidic pH, while UV region exhibits a single shift from ~282 to 322 nm.

Changes observable in the control (Ar-treated) enzyme are likely to be due to global conformational changes upon pH transition. The 324- and 352-nm bands appear in the region free from protein absorption and should be attributed to hemes and/or CuB. Although the 274 and 297 nm bands appear close to the protein 280-nm band, direction of an apparent shift is opposite to what would be expected if it arose from protonation of Tyr. Several heme transitions and charge-transfer (CT) bands of Cu3+–OH and Cu2+–imidazole are expected in this region (see below).

4. Discussion

Pm and F* are the two primary species in the peroxide reaction at high and low pH, respectively [35,36]. Both species have ferriyloxo structure of heme a3 and contain additional oxidizing equivalent at the active site. Radioactive labeling showed that Y244 in Pm is oxidized [16]. Recent ENDOR studies on F* revealed an unusual radical signal similar to the spectrum of W191• in cytochrome c peroxidase compound ES [37,38]. At the same time, Pm and F* exhibit distinct and characteristic changes in the UV region, as shown here. These changes disappear upon reduction into subsequent F species. This is the most striking in F*, which is spectrally indistinguishable from F above 400 nm [35]. It is therefore tempting to correlate observed UV changes with these additional redox centers. However, because CcO contains multiple redox cofactors and Pm lacks a good reference species (like F/F’), several possible origins must be considered before conclusive assignment can be made.

4.1. Heme a3

Pm and F species exhibit surprisingly large spectral difference in the red region. While possible causes for this have been discussed [7,39], it is not known how much the same factors could affect the UV region of the spectrum. Knowledge of UV properties of hemes below 300 nm is mostly derived from computational studies, which predicted a number of high-energy π – π* transitions sensitive to oxidation state and axial ligands [40-43]. Intensities of these transitions rely on dipole transition moments of isolated pyrrole rings and, therefore, are significantly weaker than that of B and Q bands that rely on the entire macrocycle [43]. As a result, N and L bands between 300 and 360 nm are typically the highest-energy transitions experimentally observable in heme proteins [44,45] and could contribute to the spectral changes shown in Fig. 2A. The next high-energy transition observed for model porphyrin complexes in gas phase is the M band around 225 nm [43,46] leaving a gap in the 240–300-nm region. Consistently, F and Ox show little difference in this region, suggesting that the signal observed in Pm is not likely to be significantly contributed by the heme.

4.2. CuB

Geometry and electronic configuration of CuB in each particular species will have strong influence on its UV
suggesting a CuB coupled to a loss of proton [56]. Catalytic radicals of both have relatively low oxidation potential, especially when 

$P_m$ 

different redox states [13,49]. Binding of $H_2O/OH$ of its three His ligands does not change significantly in below). $Pm$ 

$\text{Cu}^{2+} \text{dx}_2 – y_2$ orbital. Particularly relevant OH metal CT transitions, i.e. from ligand valence orbitals into $\text{Cu}^{2+}$, $d_{x_2 – y_2}$ orbital. Particularly relevant OH of such configuration are mostly defined by ligand-to- $\text{Cu}^{2+}$ does not originate from either of the two hemis. The wavelength of the positive band at 322 nm, however, is close to the reported absorption maximum of the of $\text{Trp}$ radical species [21]. While no difference spectra of $\text{Trp}$ oxidation were reported below 300 nm, the negative component at 282 nm is very close to absorption maximum of ground state $\text{Trp}$. The yield of calculated $\text{F}^*$ species in trace $b$ (Fig. 2B) is approximately 40%, if extinction of 5.5 mM$^{-1}$ cm$^{-1}$ is assumed for the 575-nm peak [69]. When this spectrum was scaled to 100% occupancy at 575 nm, the amplitude of the 322-nm band was close to that of transient $\text{Trp}^*$ [21]. Thus, there is a good match between the 322/282-nm shift and expected difference spectrum of oxidation of $\text{Trp}$. The wavelength and the intensity of the positive band, however, appear closer to those of neutral $\text{Trp}$ rather than cation $\text{TrpH}^+$, while ENDOR analysis by Rich and coworkers favored the cation species [37,38]. Published optical spectra of $\text{Trp}^*$ and $\text{TrpH}^+$ in the UV region do not allow to identify, conclusively, which form is present in $\text{CcO}$, but both spectroscopic techniques are in agreement about the oxidation of $\text{Trp}$ residues.

4.3. Origin of UV signal in $F^*$

Currently, $F^*$ cannot be generated directly without a host of side reactions involving $H_2O_2$ [63], including formation of noncatalytic radicals that will contribute to the UV region [37,64–68]. Consequently, in this study, $F^*$ was generated indirectly from $P_m$ species by lowering the pH of the sample [35]. Spectral similarity between $F^*$ and $F$ in the visible region strongly suggests that the observed 322/282 nm shift does not originate from either of the two hemis. The wavelength of the positive band at 322 nm, however, is close to the reported absorption maximum of the of $\text{Trp}$ radical species [21]. While no difference spectra of $\text{Trp}$ oxidation were reported below 300 nm, the negative component at 282 nm is very close to absorption maximum of ground state $\text{Trp}$. The yield of calculated $\text{F}^*$ species in trace $b$ (Fig. 2B) is approximately 40%, if extinction of 5.5 mM$^{-1}$ cm$^{-1}$ is assumed for the 575-nm peak [69]. When this spectrum was scaled to 100% occupancy at 575 nm, the amplitude of the 322-nm band was close to that of transient $\text{Trp}^*$ [21]. Thus, there is a good match between the 322/282-nm shift and expected difference spectrum of oxidation of $\text{Trp}$. The wavelength and the intensity of the positive band, however, appear closer to those of neutral $\text{Trp}$ rather than cation $\text{TrpH}^+$, while ENDOR analysis by Rich and coworkers favored the cation species [37,38]. Published optical spectra of $\text{Trp}^*$ and $\text{TrpH}^+$ in the UV region do not allow to identify, conclusively, which form is present in $\text{CcO}$, but both spectroscopic techniques are in agreement about the oxidation of $\text{Trp}$ residues.

4.3. Origin of UV signal in $P_m$

$\text{Tyr}$ and its specific derivative $\text{Im}$CrOH have sufficiently low oxidation potential and low $pK_a$ of phenol oxygen, so that either oxidation or deprotonation (see above) can occur. Optical shift of approximately 306/280 nm associated with deprotonation of $\text{Im}$CrOH can be deduced from published data [29,30] and is ~20 nm to red from the signal in $P_m$. While optical changes associated with oxidation of $\text{Im}$CrOH are not known, to some extent they can be anticipated. Transient UV spectrum of $\text{TyrO}^+$, reported previously, suggests significant perturbations of the 200–300-nm region upon oxidation, although it is not possible to calculate a reliable difference spectrum needed for comparison with $\text{CcO}$. Such difference spectrum was obtained experimentally and shown in Fig. 3. It can be seen in Fig. 3 that $\text{TyrO}^- → \text{TyrO}^+$ transition is associated with the optical shift from 235 to 268 nm, while the visible region shows formation of a characteristic band at 410 nm. Since $pK_a$ of $\text{TyrO}^+$ is as low as ~2 [20,70], its spectrum will show little pH sensitivity, and a shift from ~220 to ~270 nm can therefore be estimated for the
The TyrOH → TyrO* transition. In either case, oxidation of Tyr produces optical changes that are closest among aromatic amino acids to that observed in Pm. Amplitudes of signals observed for Tyr and CcO of ~10 mM−1 cm−1 are in good agreement as well.

The apparent 23-nm difference between UV shift of TyrO* and Pm may be accounted for by the presence of the covalent bond between Y244 and H240 and their binding to CuB. The electrostatic effect of a partial atomic charge of imidazole nitrogen near the ortho-carbon of phenol is similar to the effect of hydrogen bonding and deprotonation of phenol hydroxy group in the solution [54]. In fact, all reported spectra of models of the H240,Y244 site consistently show ~10-nm red shift of the phenol ring π−π* transitions from that of Tyr, compared to 19-nm red shift upon deprotonation of either compound [29,30,58].

Merging of π-orbitals at small dihedral angles between imidazole and phenol rings, like in CcO [13], will create a longer conjugated orbital, causing further red shift. A small shift in absorption of phenol moiety in response to protonation of imidazole nitrogen, significant increase in acidity of both moieties, as well as change in the acidity of imidazole upon oxidation of phenol all indicate that such electronic interactions take place [29,30,58]. Lastly, binding to CuB is likely to alter the electronic configuration of imidazole/phenol π-coupled dimer, as well as conjugation with Y244 will necessarily affect H240 → Cu ligand-to-metal CT bands, which are expected in the 300-nm region. Delocalization of the radical between imidazole and phenol creates a possibility of a metal-to-ligand CT transition, which will be equivalent to a transient formation of a Cu3+-HY− state. Intricate details of the H240,Y244,CuB site are beginning to be addressed, as more sophisticated, second-generation models become available [71,72].

The analysis presented here demonstrates that biological radicals, such as Tyr* and Trp*, can be detected and identified by their UV absorption properties even in such complex systems as CcO. This is illustrated by detection of Trp* in F*. Spectrum of Tyr* is the closest to the signal observed in Pm, although no exact match was identified. This signal is proposed to arise from oxidation of Y244 with altered UV properties due to cross-linking to CuB-bound H240.

Fig. 3. UV difference absorption spectrum of oxidation of Tyr. Glassy samples of 250 μM Tyr in 5 mM LiOH, 7 M LiCl, were oxidized at 140 K by UV output of Hg arc lamp. The spectrum shown was obtained by global exponential fitting of ~100 spectra acquired between 0.1 s and 60 min of illumination.
Efforts are currently under way to test this assignment by characterizing the UV absorption properties of corresponding model radicals.

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