Light-Induced Spectral Changes in Fully Oxidized Cytochrome *c* Oxidase in the Presence of Oxygen†

Jennifer L. Brooks, Artur Sucheta, and Ólöf Einarsdóttir*

Department of Chemistry and Biochemistry, University of California, Santa Cruz, California 95064

Received December 10, 1996; Revised Manuscript Received March 20, 1997⊗

ABSTRACT: Illumination of oxidized cytochrome oxidase with low intensity (<2 mW) light below 300 nm in the presence of oxygen causes pH-dependent spectral changes in the Soret and visible regions. The light-induced difference spectra show a peak at 438 nm and a trough at 414 nm in the Soret region and a peak at 606 nm and a shoulder at ∼577 nm in the visible region. The effect was inhibited by cyanide, suggesting the involvement of cytochrome a_3. The pH dependence indicates two titratable groups with pK_a values of 6.52 ± 0.26 and 6.85 ± 0.15. The spectral changes are analogous to those occurring upon addition of hydrogen peroxide to the fully oxidized enzyme, which results in a mixture of species with absorbance maxima at 607 and 580 nm when referenced against the oxidized enzyme. Catalase addition affected the initial onset of the spectral change and increased the rate at which the reverse reaction occurred upon termination of illumination. The data are consistent with a mechanism involving light-induced autoreduction of the binuclear center and subsequent O_2 binding, followed by the release of hydrogen peroxide and the formation of a mixture of the 607 nm and 580 nm forms.

The reduction of dioxygen to water catalyzed by cytochrome *c* oxidase has been extensively studied [see Einarsdóttir (1995) for a recent review], and spectral characteristics of the intermediates are becoming resolved. Recent flow-flash experiments on the reaction of the fully reduced bovine heart enzyme with dioxygen have shown that species with absorption maxima at ∼607 and ∼580 nm when referenced against the oxidized form are intermediates in the catalytic cycle at room temperature (Sucheta et al., 1997) and at −25 °C (Morgan et al., 1996). A counterpart of the 607 nm form of the bovine heart enzyme has been detected in other species, including cytochrome *bo* from *Escherichia coli* (Morgan et al., 1995). The 607 nm species has previously been observed during the reaction of the mixed-valence bovine heart enzyme with dioxygen at low temperature (Chance et al., 1975; Clore et al., 1980) and room temperature (Hill & Greenwood, 1983; Han et al., 1990b), but can also be generated when the resting enzyme is exposed to a mixture of CO and O_2 or substoichiometric amounts of hydrogen peroxide (Bickar et al., 1982; Wrigglesworth, 1984; Vygodina & Konstantinov, 1988; Fabian & Palmer, 1995b). The 580 nm form can be generated upon addition of excess hydrogen peroxide to the resting enzyme (Bickar et al., 1982; Fabian & Palmer, 1995a). Species with absorbance maxima at 607 and 580 nm when referenced against the oxidized enzyme are formed upon reversal of the O_2 reaction (Wikström & Morgan, 1992) and have been attributed to peroxy, P[1] ($a_3^{3+}-O^{-}-O^{-}$), and ferryl, F ($a_3^{4+}=O$), forms of cytochrome a_3, respectively. Therefore the 607 nm and 580 nm species can be produced under different conditions as long as reducing equivalents are provided, either by CO, by H_2O_2, or, in the case of the reversal of the O_2 reduction reaction, by the binuclear center (and water).

Isolated cytochrome *c* oxidase and the enzyme in whole mitochondria have been reported to be photoreduced by laser light in the presence and absence of oxygen (using specific excitation wavelengths in the Soret and visible regions) during resonance Raman experiments (Adar & Yonetani, 1978; Salmeen et al., 1978; Adar & Erecinska, 1979; Babcock & Salmeen, 1979; Ogura et al., 1985). Changes in the Soret and visible spectra of isolated cytochrome *c* oxidase, indicative of photoreduction, also occur in the absence of oxygen when the enzyme is irradiated with a quartz/iodine lamp (Nicholls & Chanady, 1981). However, little is known about the effect of light on the UV visible spectra of oxidized cytochrome *c* oxidase in the presence of oxygen.

Photoreduction has also been reported to induce turnover (Ogura et al., 1985), and resonance Raman experiments have shown that a transient intermediate, $Fe_{a3}^{3+}-OH$, that is formed during the reaction of reduced cytochrome oxidase with dioxygen (Han et al., 1990a) can be generated upon exposing the oxidized enzyme to 413.1 nm laser light in the presence of oxygen (Han et al., 1989). This suggests that other intermediates generated during dioxygen reduction by cytochrome oxidase may be obtained upon illumination in the presence of dioxygen.

In this study, we report pH-dependent light-induced changes in the Soret and visible absorption spectra of oxidized cytochrome *c* oxidase induced by illumination with a deuterium lamp in the presence of oxygen. The spectral

† This work was supported by National Institutes of Health Grant GM45888.

* Author to whom correspondence should be addressed. E-mail: olof@chemistry.ucsc.edu. Fax: 408-459-2935.

⊗ Abstract published in *Advance ACS Abstracts,* May 1, 1997.

[1] Abbreviations: CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; TRICINE, *N*-[tris(hydroxymethyl)methyl]glycine; P, a form of the enzyme in which both cytochrome a_3 and Cu_B are oxidized and cytochrome a_3 has an absorbance maximum at ∼607 nm when referenced against its oxidized state; F, a form of the enzyme in which cytochrome a_3 has an absorbance maximum at ∼580 nm when referenced against its oxidized state.

S0006-2960(96)03032-2 CCC: $14.00

shifts are the same as those observed when hydrogen peroxide is added to the oxidized enzyme, which produces a mixture of species with maxima at 580 nm and 607 nm when referenced against the oxidized enzyme.

MATERIALS AND METHODS

Cytochrome oxidase was purified from fresh bovine hearts according to Yoshikawa et al. (1977). The concentrated enzyme was stored at −80 °C in 0.1 M sodium phosphate, pH 7.4, for no more than 6 months. Enzyme concentration was determined based on an extinction coefficient of 158 mM^{-1} cm^{-1} at 420 nm for the oxidized enzyme (Fabian & Palmer, 1995b). The enzyme was diluted into the appropriate buffer and filtered through a 0.2 μm filter prior to exposing it to light. The effect of light on the Soret and visible spectra of cytochrome oxidase was probed by continuously illuminating an aerobic enzyme solution in a stirred temperature-controlled quartz cuvette with a Hewlett-Packard (Palo Alto, CA) 8452 deuterium lamp (<2 mW). The shutter remained open for an hour during which spectra were recorded every 25 s. The effect of light on the spectra as a function of pH was probed as described in the text and figure legends.

The 607 nm complex and the 580 nm form were generated as described by Fabian and Palmer (1995a). Briefly, the 607 nm form was obtained by incubating a 3.8 μM solution of cytochrome *c* oxidase with carbon monoxide for ∼2 min in the presence of air. The 580 nm species was formed by adding H_2O_2 at a final concentration of 1 mM to a 3.8 μM enzyme solution. Both forms were generated at pH 8.

The light-induced reaction in the presence of catalase was performed under the same conditions as in its absence (see Figure 1 for details), except 1 nM catalase was present during the illumination. To explore the effect of light on oxidase with cyanide present, neutralized KCN was added to 5 μM cytochrome oxidase at a final concentration of 30 mM. Only after absorbance changes at 428−420 nm, indicative of cyanide binding, were complete (∼12 h), was the light-induced reaction initiated. The final difference spectrum observed prior to light exposure (a maximum at 434 nm and minimum at 412 nm) was essentially identical to that reported earlier (Van Buuren et al., 1972).

Data analysis was performed on a Power Macintosh (Cupertino, CA) using Kaleidagraph (Synergy Software, Reading, PA) and Matlab (The MathWorks, Inc., Natick, MA) software.

RESULTS

Figure 1 shows a series of Soret and visible absorption spectra obtained every 25 s during 1 h illumination of oxidized cytochrome oxidase in the presence of oxygen. The arrows indicate the direction of the shift. A 6 nm shift from 420 nm to 426 nm (Figure 1a) and an increase at 607 nm (Figure 1b) are clearly apparent. The red shift in the Soret spectra is reflected in the corresponding time-resolved difference spectra (after minus before illumination) (Figure 1c), which show a trough at 414 nm and a peak at 438 nm after 1 h of illumination. The maximum in the difference spectra at 200 s after illumination shifts from 444 nm to a final peak at 438 nm, indicating the formation of more than one species. The difference spectra in the visible region (Figure 1d) show an increase at 607 nm with a shoulder

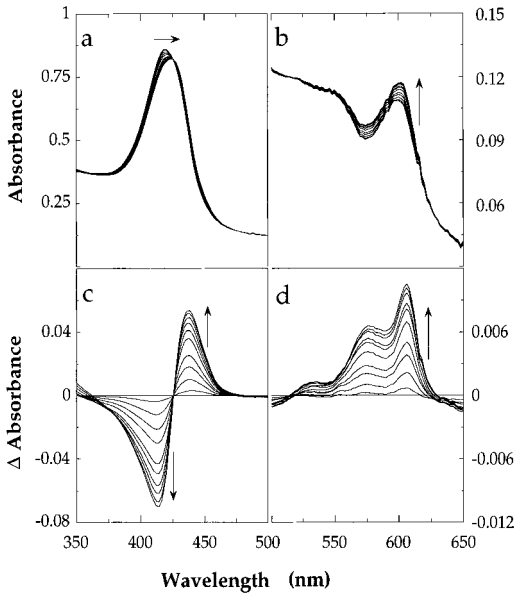


FIGURE 1: (a and b) Light-induced absorption spectra of resting cytochrome oxidase in the Soret (a) and visible (b) regions. The spectra were recorded at 25, 200, 600, 800, 1200, 1500, 2100, 2700, 3300, and 3600 s during 1 h illumination of the enzyme with a deuterium lamp (<2 mW). (c and d) Light-induced absorption difference spectra in the Soret (c) and visible (d) regions (after minus before illumination). The spectra were recorded at the same time points as in panels a and b. The cytochrome oxidase concentration was 5 μM in 0.1 mM HEPES/0.1 M KCl, pH 7.5, 24 °C.

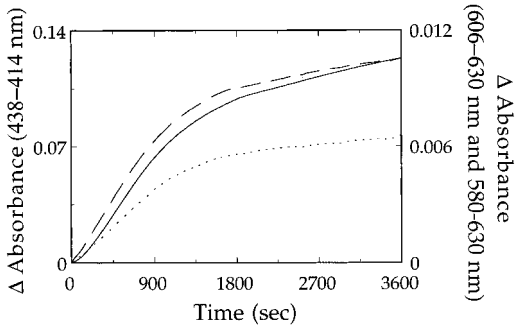


FIGURE 2: Light-induced kinetic profiles. The change in the absorbance at 438−414 nm (solid line), 606−630 nm (dashed line), and 580−630 (dotted line) was followed as a function of time every 25 s during 1 h light exposure. The concentration of the enzyme and conditions were the same as those listed in Figure 1.

appearing at ∼577 nm. The time courses of the spectral changes in the Soret region (438−414 nm) and visible region (606−630 nm and 580−630 nm) are sigmoidal, supporting the existence of one or more intermediates different from the reactants and products (Figure 2). The light-induced spectral shifts at 414 and 438 nm, the apparent isosbestic point at 426 nm, and the peak at 607 nm and the shoulder at ∼577 nm in the visible region (Figure 1) are similar to those observed by Fabian and Palmer (1995b) when substoichiometric amounts of hydrogen peroxide were added to the oxidized cytochrome oxidase at pH 8. These spectral characteristics were attributed to a mixture of the 607 nm and 580 nm species. The light-induced spectral changes are also similar to those reported by Nicholls and Chanady (1981) when anaerobic photoreduced cytochrome oxidase was exposed to oxygen.

Illumination up to ∼2 h resulted in the same spectral characteristics as those described above for 1 h. However,

upon longer illumination (up to 9.5 h), the maximum at 438 nm in the difference spectrum shifted to 444 nm and decreased in intensity, while the trough at 414 nm increased in magnitude with time. In the visible region, the 607 nm band shifted toward 600 nm while the shoulder at 577 nm remained unchanged.

When illumination was terminated after 1 h and the enzyme was allowed to relax in the dark for an additional hour, the trough at 414 nm in the Soret difference spectrum decreased significantly in intensity, and the peak at 438 nm shifted to 434 nm accompanied by a significant loss in intensity (not shown). In the visible region, the peak at 607 nm in the difference spectrum disappeared, but an absorption band with a maximum at 580 nm remained. These difference spectra are analogous to those of the 580 nm species alone (Fabian & Palmer, 1995a). Longer relaxation (up to 12 h) resulted in the disappearance of the peak at 434 nm and the formation of a negative shoulder at ~430 nm. Simultaneous spectral changes in the visible region resulted in distortion of the base line and the 580 nm peak, indicating that some denaturation might have taken place.

The light-induced spectral changes did not occur when the enzyme was fully reduced and were practically eliminated when the oxidized enzyme was pretreated with cyanide. This suggests that cytochrome a_3 is involved in the light-induced effect. Increasing the oxygen concentration did not alter the spectral characteristics or the rate of the induced changes, implying that the effect is already saturated at 20% oxygen. Decreasing the light intensity with neutral density filters resulted in a decreased amplitude and rate of the spectral change, while the amplitude was smaller and the rate increased as the concentration of oxidase was decreased. The activity of the enzyme, measured by the rate of cytochrome *c* oxidation, was unchanged after 1 h of illumination. This is in agreement with previous studies, which found that O_2 consumption in purified oxidase and uncoupled mitochondria was unaffected by white light, while O_2 consumption in coupled mitochondria in the presence of ADP was accelerated (Kato et al., 1981).

The wavelengths responsible for the light-induced spectral changes were investigated by using various filters to restrict the probe beam to different spectral regions. The enzyme was illuminated for 1 h in the absence and presence of a filter. Four different filters were used with the region of transmitted light: (1) greater than 300 nm (Schott filter), (2) between 260 nm and 410 nm and greater than 680 nm (Corning filter), (3) between 230 nm and 410 nm and greater than 690 nm (U330 filter), and (4) less than 230 nm (200-b-d-filter). After correcting for the percent transmittance of each filter, the 200-b-d filter gave a difference spectrum similar to that obtained in the absence of a filter, while the difference spectra using the Schott, Corning, and U330 filters were of much smaller magnitude (<5% of the spectrum obtained in the absence of a filter). This indicates that at the light intensity used (<2 mW), only wavelengths below 230 nm are effective in causing the observed spectral shifts.

pH Effects. Light-induced spectral changes were followed as a function of pH in the Soret and visible regions. Because oxidized cytochrome oxidase undergoes a pH-induced spectral shift in the absence of light (Papadopoulos et al., 1991), the enzyme was diluted into a buffer at the appropriate pH and monitored until the pH-induced shift was complete prior to exposing the sample to light. Analysis of the titration

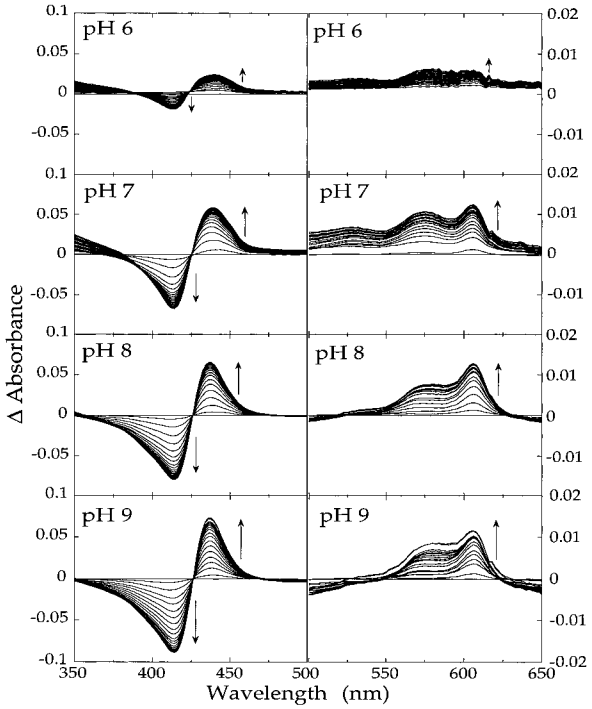


FIGURE 3: Light-induced absorption difference spectra as a function of pH. Before illumination, cytochrome oxidase was allowed to equilibrate in the appropriate pH buffer until the position of the maximum of the Soret band remained unchanged. Incubation times required were between 1 and 3 h. The difference spectra were recorded every 200 s (starting at 25 s) during 1 h illumination. The concentration of the enzyme and conditions were the same as those listed in Figure 1.

profile of the Soret band maximum before illumination gave an apparent pK_a of 7.8 ± 0.2, in agreement with that reported earlier (Papadopoulos et al., 1991). A significant pH dependence of the light-induced shift was observed in both the Soret and visible bands, with larger spectral changes occurring at higher pHs (Figure 3). Assuming a single titratable group, a nonlinear least squares fit of the change in absorbance at 438−414 nm and 606−630 nm as a function of pH after 1 h of illumination gave an apparent pK_a (± standard deviation) of 6.85 ± 0.15 (Figure 4, solid and dashed lines, respectively). This pH dependence is similar to that reported for the Soret band (Bickar et al., 1982) and the 607 nm peak (Vygodina & Konstantinov, 1988, 1989) upon addition of hydrogen peroxide to the oxidized enzyme. A pK_a of 6.52 ± 0.26 was obtained when the spectral change was monitored at 580−630 nm (Figure 4, dotted line).

The effects of concentration and protonation state of the buffer on the light-induced spectral change were investigated using buffers with a wide range of pK_as: MES (pK_a = 6.1), HEPES (pK_a = 7.55), TRICINE (pK_a = 8.15), and CHES (pK_a = 9.5). At a constant pH, a larger spectral change was observed as the pK_a decreased or the concentration of the buffer was increased, suggesting that the unprotonated form of the buffer plays a role in producing the spectral change.

Light-Induced vs H_2O_2-Induced Spectral Changes. Figure 5a shows a comparison between the light-induced visible difference spectrum (solid line) and the visible difference spectrum obtained after adding hydrogen peroxide to the oxidized enzyme in the absence of light (dashed line). The latter was recorded 20 min after addition of peroxide at a 2:1 molar ratio to the enzyme (3.8 μM). At this H_2O_2 concentration, a mixture of two species with maxima at 607

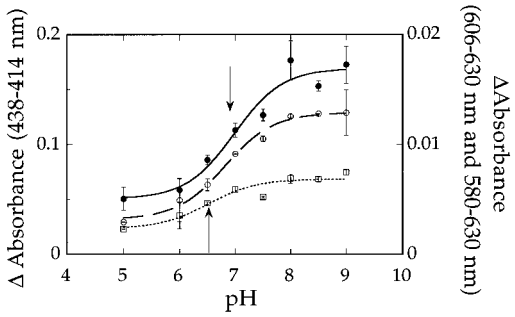


FIGURE 4: Titration profiles of the light-induced spectral changes as a function of pH. The experiments were performed as described in Figures 1 and 3. The data are the average ΔA values (after minus before light) at the indicated wavelengths of the difference spectra. Two or three experiments were performed for each pH . The curves represent fits to the data according to the formula $\Delta A_{max} = [m1 + m2(10^{pH-pK_a})]/(10^{pH-pK_a} + 1)$ using the Levenberg−Marquardt algorithm, where $m1$ and $m2$ are the lower and upper limits of the titration profiles, respectively. The ΔA(438−414 nm) experimental values are shown as filled circles and the fit as a solid line, the ΔA(606−630 nm) experimental values are shown as open circles and the fit as a dashed line, and the ΔA(580−630 nm) experimental values are represented by open squares and the fit as a dotted line. Error bars indicate the standard deviation for the points averaged. The arrows indicate the two pK_as.

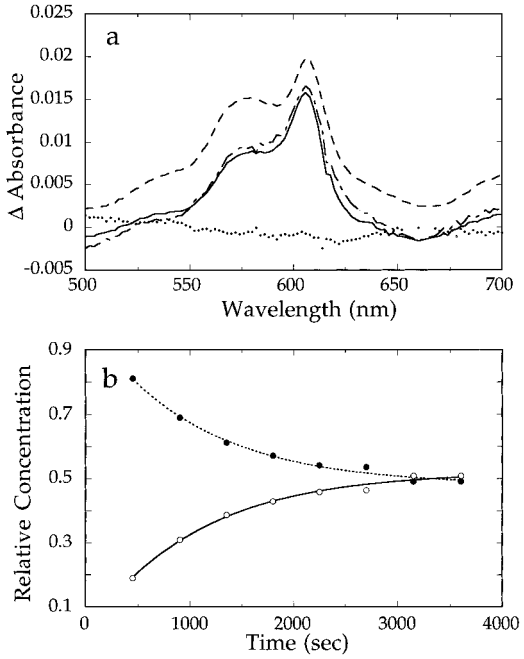


FIGURE 5: (a) Comparison of the light-induced (solid line) and H_2O_2-induced (dashed line) visible difference spectra. The reference spectrum is that of the oxidized enzyme. The light-induced difference spectrum (solid line) was recorded after 1 h illumination. The H_2O_2-induced visible difference spectrum (dashed line) was recorded 20 min after addition of H_2O_2 at a 2:1 molar ratio to the enzyme. The dashed−dotted line is the sum of spectra of the pure 607 nm and 580 nm forms in a 1:1 ratio, prepared as described under Materials and Methods. The difference between the light-induced spectrum and the 1:1 607 and 580 nm forms is the dotted line along the zero line. (b) Relative fractions of the 607 nm and 580 nm forms as a function of time. The concentration of cytochrome oxidase was 3.8 μM in 0.1 M HEPES/0.1 M KCl at pH 8.5, 24 °C. The relative populations were obtained by deconvoluting the light-induced difference spectra obtained at different times during illumination using the spectra of pure 607 nm and 580 nm forms. Filled and open circles represent the relative concentrations of the 607 nm and 580 nm forms, respectively. The dotted and solid lines are an exponential fit to relative amounts of the 607 nm and 580 nm forms, respectively.

and 580 nm is formed. There are striking similarities between the light-induced and the H_2O_2-induced difference spectra (Figure 5a). Both show a peak at 607 nm, a shoulder at ~577 nm, and a trough at 650 nm. Deconvolution of the light-induced difference spectrum (obtained after 1 h illumination) using the difference spectra of separately prepared 607 nm and 580 nm forms indicated that equal amounts of the two species were present. Figure 5a (dot−dashes) shows a fit to the light-induced difference spectrum (solid line) using equal amounts of the 607 and 580 nm forms. The residual spectrum (the difference between the data and the fit) is shown as the dotted line along the zero line. Using $\epsilon_{(420)} = 158$ mM^{-1} cm^{-1} for the fully oxidized enzyme (Fabian & Palmer, 1995b) and $\Delta\epsilon_{(607-630)} = 11$ mM^{-1} cm^{-1} and $\Delta\epsilon_{(582-630)} = 5.3$ mM^{-1} cm^{-1} for the 607 nm form and 580 nm species, respectively (Wikström & Morgan, 1992), we estimated that ~40% of the enzyme was converted to the 607 nm and 580 nm forms at pH 8.5, while 60% remained in the oxidized form. At a lower pH, the amount of the 580 nm species increased at the expense of the 607 nm form. The reason for the low yield of the two species presumably is due to limitation of the available source light intensity. However, it should be noted that the conversion of the oxidized enzyme to the 607 nm and 580 nm species upon exposure to a CO/O_2 mixture and hydrogen peroxide, respectively, was only 50%.

The relative contributions of the 607 nm and 580 nm forms to the light-induced spectra as a function of illumination time were also determined. At pH 8.5, exposure to light led to the conversion of the oxidized enzyme to the 607 nm form, followed by partial conversion to the 580 nm species (Figure 5b). The decay of the 607 nm complex and the formation of the 580 nm species both exhibit exponential behavior, and as indicated above, after 1 h of illumination equal amounts of the 607 nm and the 580 nm species were present (Figure 5b).

Catalase Effects. In view of the similarities between the light-induced and the H_2O_2-induced visible difference spectra, we investigated the effect of catalase on the extent of the light-induced spectral changes and on the rate of reversion of the spectral changes upon termination of light exposure. Catalase is responsible for the conversion of hydrogen peroxide into water and molecular oxygen and is known to reverse the effect that hydrogen peroxide has on cytochrome oxidase (Bickar et al., 1982; Vygodina & Konstantinov, 1988). With catalase present in the reaction mixture, the light-induced difference spectrum after 1 h of illumination is different from that observed in its absence, with a maximum at ~443 nm and a minimum at 414 nm in the Soret region (Figure 6a) and a maximum at ~606 nm in the visible region (Figure 6b). The prominent shoulder at ~577 nm in the light-induced difference spectrum in the absence of catalase is significantly reduced in the presence of catalase. These results suggest that hydrogen peroxide generated in the bulk solution as a result of the illumination is rapidly decomposed by catalase. When catalase is added after 1 h illumination, the relaxation of the light-shifted cytochrome oxidase was found to occur faster than in the absence of catalase, with a final spectrum similar, although not identical, to that observed prior to illumination. The addition of superoxide dismutase did not have any effect on the amplitude or the rate of the light-induced spectral changes.

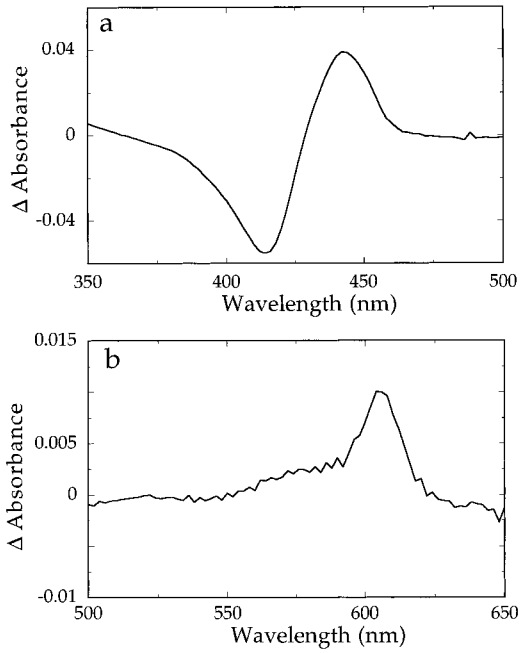
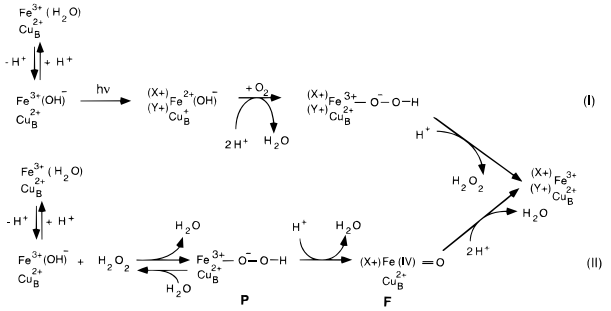


FIGURE 6: Light-induced spectral changes in the presence of catalase. The absorption difference spectra in the Soret (a) and visible region (b) were obtained after 2 h of illumination of the enzyme in the presence of catalase. The enzyme concentration and the experimental conditions were the same as those listed in Figure 1.

Scheme 1



DISCUSSION

A model explaining the light-induced effect on cytochrome oxidase must account for the following observations: (1) light-induced difference spectra with maxima at 438 nm and 607 nm, a trough at 414 nm and a shoulder at ~577 nm, reflecting the formation of a mixture of the 607 nm and 580 nm species, (2) elimination of the effect by cyanide, (3) pH dependence associated with titratable groups with apparent pK_a values of 6.52 and 6.85, (4) larger spectral changes when the pK_a of the buffer is lowered or the concentration of the buffer is increased, and (5) the effect of catalase on the light-induced difference spectra and the faster relaxation back to the "oxidized" enzyme in its presence.

Scheme 1 represents a possible pathway to explain the light-induced effect on cytochrome oxidase. For clarity, only cytochrome a_3 and Cu_B are represented. The mechanism involves two reactions, I and II. In reaction I, H_2O_2 is generated which is used in reaction II. To account for the larger spectral changes observed at higher pHs and in buffers with lower pK_a, the original resting oxidized enzyme is proposed to be in a slow equilibrium with a deprotonated form of the enzyme with the buffer serving as the proton acceptor. Illumination of the deprotonated species causes autoreduction of cytochrome a_3, and possibly Cu_B, resulting in formation of $Fe^{2+}(OH)^-Cu_B^+$. This is reflected by a Soret maximum of 444 nm in the difference spectrum obtained in the initial stages (after 200 s illumination) of the experiment. The reducing equivalents are most likely provided by protein residues, represented by X and Y in Scheme 1. Oxygen then binds to cytochrome a_3, and the binuclear center is oxidized, resulting in the formation of a species with a maximum at 607 nm in the light-induced difference spectrum. This species is represented in Scheme 1 as a peroxy species, (X+)-(Y+)$Fe^{3+}-O^--O-H$ Cu_B^{2+}, where (X+) and (Y+) are amino acid radicals. We do not know the protonation state of the peroxy form, but in view of the pK_a of 6.85 observed in the Soret region and at 606−630 nm, we suggest that one of the four histidine ligands of the binuclear center may be deprotonated upon formation of this complex (Fabian & Palmer, 1995b). A further uptake of one proton leads to the formation of the doubly protonated complex, followed by the release of H_2O_2 and the formation of a species, (X+)-(Y+)Fe^{3+} Cu_B^{2+}, 2 oxidizing equivalents above the original oxidized enzyme.

In reaction II, the H_2O_2 released in reaction I reacts with the oxidized deprotonated enzyme. This reaction involves the initial formation of a 607 nm peroxy species, $Fe^{3+}-O^--O-H$ Cu_B^{2+}, denoted as P, and the release of water (Konstantinov et al., 1992). The peroxy species in reaction I and P are assumed to have analogous absorption spectra, both with an absorbance maximum of 607 nm in the oxidized difference spectrum. The protonation state of the P form agrees with that reported previously (Konstantinov et al., 1992; Mitchell et al., 1992). Subsequent one-electron reduction converts the P form into a 580 nm species, depicted as a ferryl, F, $Fe^{4+}=O$ Cu_B^{2+}, with a reducing equivalent provided by the protein moiety. This step also requires a proton, reflected by the pH dependence at 580−630 nm (pK_a = 6.52), and water is released. The ferryl form finally decays to the (X+)(Y+)Fe^{3+} Cu_B^{2+} form (see below).

The binding of cyanide to cytochrome a_3 prevents the light-induced spectral changes, supporting the involvement of this metal center. A species with peaks at 425 nm and ~600 nm in the absolute spectra, similar to that observed here, has previously been reported upon illumination of anaerobic cytochrome oxidase with light between 400 and 610 nm, followed by exposure to air (Nicholls & Chanady, 1981). This form of cytochrome oxidase was not characterized further, although its similarity to the oxygenated dithionite-reduced enzyme was noted.

The structures of the 607 nm and 580 nm species have generally been attributed to peroxy and ferryl forms, respectively, but recent studies have questioned these assignments (Proshlyakov et al., 1994; Fabian & Palmer, 1995b). However, room temperature flow-flash studies in our laboratory (Sucheta et al., 1997) of the reaction of the reduced cytochrome oxidase with dioxygen agree with previous assignments, and these are supported by similar studies carried out at −25 °C (Morgan et al., 1996). In view of these results, we suggest that the peroxy, P, and the ferryl, F, structures depicted in Scheme 1 correspond to the 607 nm and 580 nm species, respectively.

After termination of light exposure, the initial autoreduction step in reaction I is prevented, thereby inhibiting both reactions. During the first hour of relaxation, the peak at

607 nm disappears, but a peak at 580 nm remains. In the Soret region, the peak at 438 nm is shifted to 434 nm and decreases significantly in intensity together with the 413 nm trough. These results indicate that the accumulated peroxy species is rapidly converted to the ferryl form in reaction II. This is consistent with the observation that the 607 nm species made by the addition of H_2O_2 decays to the 580 nm species (Wrigglesworth, 1984; Vygodina & Konstantinov, 1988, 1989; Fabian & Palmer, 1995b).

Scheme 1 also accounts for the effects of catalase. Catalase removes the H_2O_2 produced in reaction I, thus preventing reaction II from taking place. According to Scheme 1, the light-induced difference spectrum in the presence of catalase would be expected to be a mixture of the species produced in reaction I, and deconvolution of the spectra shows this to be the case.

When catalase is added after termination of illumination, the H_2O_2 produced in reaction I is removed, causing a faster relaxation back to the "oxidized state". This is reflected by the essentially flat visible difference spectrum obtained in the presence of catalase 0.5 h after termination of light exposure (referenced versus the spectrum of the oxidized enzyme before exposure to light). As pointed out above, the first step leading to the autoreduction is prevented upon termination of illumination, and reaction I proceeds to the right until all the intermediates are exhausted, with $(X+)$-$(Y+)Fe^{3+}$ Cu_B^{2+} as the final product. For reaction II, the termination of illumination and removal of hydrogen peroxide by catalase reverses the effect of hydrogen peroxide with the return of the enzyme toward the "oxidized" form (Bickar et al., 1982; Vygodina & Konstantinov, 1988). This could be accomplished by the uptake of two protons and abstraction of an electron, presumably from protein residue Y, converting the F form, $(X+)Fe(IV)=O$ Cu_B^{2+}, to the $(X+)(Y+)Fe^{3+}$ Cu_B^{2+} species, and the decay of the peroxy form to the original oxidized state by the reversal of the oxidized-to-peroxy reaction. This type of mechanism is depicted in Scheme 1. Although we have not directly demonstrated the reversibility of the oxidized-to-peroxy step in reaction II, the conversion of the $(X+)(Y+)Fe^{3+}-O^{-}-O-H$ Cu_B^{2+} form to the $(X+)(Y+)Fe^{3+}$ Cu_B^{2+} species (the last step in reaction I), with the release of hydrogen peroxide, provides strong argument for it occurring. The reversal of the oxidized-to-peroxy reaction is also supported by previous studies (Bickar et al., 1982) which showed that the binding of H_2O_2 to cytochrome oxidase is reversed by catalase and hydrogen peroxide is released. The small differences observed in the Soret region between the initial oxidized resting enzyme and that observed following relaxation in the presence of catalase presumably reflect minor spectral differences between the former and the $(X+)(Y+)Fe^{3+}$ Cu_B^{2+} species. Alternatively, the $(X+)Fe(IV)=O$ Cu_B^{2+} intermediate could decay to the original oxidized state, if both the steps in reaction II are reversible. However, the reversibility of the peroxy-to-oxyferryl compound under our conditions and those reported previously (Bickar et al., 1982; Vygodina & Konstantinov, 1988) remains to be shown.

The nature of the light-activated reductant is unclear at this point. Rotenone inhibits electron transfer from flavin mononucleotide to coenzyme Q, but its addition had no effect on the light-induced spectra, excluding the possibility of NADH dehydrogenase contaminant (Adar & Yonetani, 1978). Furthermore, flavin contaminants were excluded based on fluorescence measurements. The identity of the two protein residues, X and Y, which may represent the light-activated reductants is unknown, but tyrosine 244 at the binuclear site (Tsukihara et al., 1995) may be involved, since tyrosine radicals are known to play a role in a variety of biological electron transfer reactions (Barry, 1993).

In conclusion, we have investigated the effects of light on oxidized cytochrome *c* oxidase in the presence of oxygen. Illumination of oxidized cytochrome oxidase with a deuterium lamp in the presence of oxygen causes spectral changes in both the Soret and visible regions that are consistent with the formation of 607 nm and 580 nm species, both of which were recently shown to be intermediates in the catalytic cycle of the fully reduced oxidase with dioxygen at room temperature (Sucheta et al., 1997). The kinetic behavior of the 607 and 580 nm species formed upon illumination indicates that the 607 nm species is first produced, and then is converted to the 580 nm form (Figure 5b), consistent with our flow-flash results (Sucheta et al., 1997). The conversion to the 580 nm form following termination of illumination may present an alternative method to prepare this species. The physiological relevance of the light-shifted enzyme remains unknown, but the fact that the light-induced species mimic those of P and F, postulated intermediates in the catalytic cycle of cytochrome *c* oxidase (Morgan et al., 1996; Sucheta et al., 1997), may provide further insight into the mechanism of the dioxygen reduction reaction.

REFERENCES

Adar, F., & Yonetani, T. (1978) *Biochim. Biophys. Acta 502*, 80−86.

Adar, F., & Erecinska, M. (1979) *Biochemistry 18*, 1825−1829.

Babcock, G. T., & Salmeen, I. (1979) *Biochemistry 18*, 2493−2498.

Barry, B. A. (1993) *Photochem. Photobiol. 57*, 179−188.

Bickar, D., Bonaventura, J., & Bonaventura, C. (1982) *Biochemistry 21*, 2661−2666.

Chance, B., Saronio, C., & Leigh, J. S., Jr. (1975) *J. Biol. Chem. 250*, 9226−9237.

Clore, M. G., Andréasson, L.-E., Karlsson, B. G., Aasa, R., & Malmström, B. (1980) *Biochem. J. 185*, 139−154.

Einarsdóttir, O. (1995) *Biochim. Biophys. Acta 1229*, 129−147.

Fabian, M., & Palmer, G. (1995a) *Biochemistry 34*, 1534−1540.

Fabian, M., & Palmer, G. (1995b) *Biochemistry 34*, 13802−13810.

Han, S., Ching, Y.-C., & Rousseau, D. L. (1989) *J. Biol. Chem. 264*, 6604−6607.

Han, S., Ching, Y.-C., & Rousseau, D. L. (1990a) *Nature 348*, 89−90.

Han, S., Ching, Y.-C., & Rousseau, D. L. (1990b) *J. Am. Chem. Soc. 112*, 9445−9451.

Hill, B. C., & Greenwood, C. (1983) *Biochem. J. 215*, 659−667.

Kato, M., Shinzawa, K., & Yoshikawa, S. (1981) *Photobiochem. Photobiophys. 2*, 263−269.

Konstantinov, A. A., Capitanio, N., Vygodina, T. V., & Papa, S. (1992) *FEBS Lett. 312*, 71−74.

Mitchell, R., Mitchell, P., & Rich, P. R. (1992) *Biochim. Biophys. Acta 1101*, 188−191.

Morgan, J. E., Verkhovsky, M. I., Puustinen, A., & Wikström, M. (1995) *Biochemistry 34*, 15633−15637.

Morgan, J. E., Verkhovsky, M. I., & Wikström, M. (1996) *Biochemistry 35*, 12235−12240.

Nicholls, P., & Chanady, G. A. (1981) *Biochem. J. 194*, 713−720.

Ogura, T., Yoshikawa, S., & Kitagawa, T. (1985) *Biochemistry 24*, 7746−7752.

Papadopoulos, P. G., Walter, S. A., Li, J., & Baker, G. M. (1991) *Biochemistry 30*, 840−850.

Proshlyakov, D. A., Ogura, T., Shinzawa-Itoh, K., Yoshikawa, S., Appelman, E. H., & Kitagawa, T. (1994) *J. Biol. Chem. 269*, 29385−29388.

Salmeen, I., Rimai, L., & Babcock, G. (1978) *Biochemistry 17*, 800−806.
Sucheta, A., Georgiadis, K. E., & Einarsdóttir, O. (1997) *Biochemistry 36*, 554−565.
Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., & Yoshikawa, S. (1995) *Science 269*, 1069−1074.
Van Buuren, K. J. H., Nicholls, P., & Van Gelder, B. F. (1972) *Biochim. Biophys. Acta 256*, 258−276.
Vygodina, T. V., & Konstantinov, A. A. (1988) *Ann. N.Y. Acad. Sci. 550*, 124−138.
Vygodina, T., & Konstantinov, A. (1989) *Biochim. Biophys. Acta 973*, 390−398.
Wikström, M., & Morgan, J. E. (1992) *J. Biol. Chem. 267*, 10266−10273.
Wrigglesworth, J. M. (1984) *Biochem. J. 217*, 715−719.
Yoshikawa, S., Choc, M. G., O'Toole, M. C., & Caughey, W. S. (1977) *J. Biol. Chem. 252*, 5498−5508.

BI9630321