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# Proton Pumping Activity and Visible Absorption and Resonance Raman Spectra of a *cao*-Type Cytochrome *c* Oxidase Isolated from the Thermophilic Bacterium *Bacillus* PS3†

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ABSTRACT: Cytochrome *c* oxidase having heme O in addition to heme C and heme A (cytochrome *cao*) [Sone, N., & Fujiwara, Y. (1991) *FEBS Lett. 288*, 154–158] was isolated from a thermophilic bacterium, *Bacillus* PS3, grown under slightly air-limited conditions. Cytochrome *cao* could oxidize yeast cytochrome *c* and *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine twice as fast as cytochrome $caa_3$, which this organism yielded under normal growing conditions. Cytochrome *cao* also pumped protons upon cytochrome *c* oxidation in a way similar to cytochrome $caa_3$. Binding of cyanide to cytochrome *cao* caused spin-state conversion of heme O at the binuclear center and seriously inhibited its physiological activity. A low $K_i$ value (0.4 $\mu$M) for cyanide was found to be mainly due to a small "off" constant. Resonance Raman spectra of cytochrome *cao* bore close resemblance to those of cytochrome $caa_3$ in both oxidized and reduced states, although the formyl stretching ($\nu_{CH=O}$) band was absent. The Fe–histidine stretching ($\nu_{Fe-His}$) and Fe–CO stretching ($\nu_{Fe-CO}$) frequencies of cytochrome *cao* were very close to those seen for cytochrome $caa_3$, but were distinct from those of hemoglobin and peroxidases, suggesting that the protein structure in the vicinity of heme O resembles that of the heme $a_3$ moiety of cytochrome $caa_3$.

A variety of terminal oxidases, including $aa_3$-type, *d*-type, and *o*-type cytochromes, have been purified from aerobic and facultative aerobic bacteria (Poole, 1988; Anraku, 1988; Sone, 1990). We reported that the Gram-positive, spore-forming thermophilic bacterium *Bacillus* PS3 shows a different pattern of cytochrome composition depending on the aeration conditions (Sone et al., 1983). The PS3 cells cultured under slightly air-limited conditions showed the mitochondrion-like cytochrome pattern composed of cytochromes *a*, *b*, and *c*, which was similar to that of the PS3 cells cultured under highly-aerated conditions, but its CO-binding cytochrome was *o*-type instead of $a_3$-type. From membranes of these cells, a new type of cytochrome was purified by almost the same procedure as used for the purification of cytochrome $caa_3$ from highly-aerated cells. On gel electrophoresis in the presence of dodecyl sulfate, the new cytochrome gave four bands at the same positions as those of cytochrome $caa_3$, but it contained heme O in addition to hemes C and A (Sone & Fujiwara, 1991). Heme O is a new kind of heme found recently as the chromophore of a quinol oxidase (cytochrome *bo*) in *Escherichia coli* (Puustinen & Wikstrom, 1991), and has been characterized to have a hydroxyethylfarnesyl side chain at position 2 like heme A but a methyl group at position 8 instead of a formyl group (Wu et al., 1992). Therefore, the new cytochrome was interpreted as cytochrome *cao*, in which heme A at the cytochrome $a_3$ center of cytochrome $caa_3$ is replaced

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with heme O (Sone & Fujiwara, 1991). Similar cytochrome oxidases probably having heme O have been found in alkalophilic *Bacillus* (Qureshi et al., 1990) and *Acetobacter aceti* (Matsushita et al., 1992), suggesting that replacement of heme A by heme O occurs rather frequently. Moreover, the homology of *E. coli* quinol-oxidizing cytochrome *bo* with mitochondrial and bacterial cytochrome (*c*)*aa*$_3$-type oxidases has been confirmed in protein sequence analysis (Chepuri et al., 1990), and spectroscopic characteristics of their binuclear centers (heme A/O–Cu$_B$), where reduction of dioxygen is catalyzed (Salerno et al., 1990; Hill et al., 1992), are also alike (Wang et al., 1993).

Here we report resonance Raman spectra of cytochrome *cao* and discuss effects of heme *o* substitution for heme *a*$_3$ on the oxidase and proton pumping activities. Comparison of the present data for PS3 cytochrome *cao* with those previously obtained for PS3 cytochrome *caa*$_3$ (Sone & Yanagita, 1992; Sone & Hinkle, 1982; Ogura et al., 1984) clearly indicates that heme O at the binuclear center behaves quite similar to heme A. Analyses of the overall reaction rate and cyanide inhibition indicate that cytochrome *cao* oxidizes cytochrome *c* and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD)[1] faster and in more cyanide-sensitive manner than cytochrome *aa*$_3$ (Nicholls & Sone, 1984; Sone & Nicholls, 1985).

## MATERIALS AND METHODS

*Materials.* PS3 cytochrome *caa*$_3$ was prepared as described previously (Sone & Yanagita, 1982; Sone, 1986). Cytochrome *cao* was obtained from PS3 cells grown under slightly air-limited conditions (Sone & Fujiwara, 1991). The method to isolate cytochrome *cao* from membranes of the PS3 cells was principally the same as that used to isolate cytochrome *caa*$_3$ (cytochrome *c* oxidase) from cells cultured with vigorous aeration (Sone, 1986). Yeast cytochrome *c* of *Saccharomyces cerevisiae* (type VIII) was purchased from Sigma Chemicals (St. Louis, MO), DEAE-Fractogel (Toyopearl) from Toso Co. (Tokyo), Q-Sepharose from Pharmacia (Upsalla), valinomycin from Boehringer (Manheim), and low molecular weight protein standards from Bio-Rad (Richmond, CA).

*Reconstitution of Proteoliposomes and Measurement of Proton Pumping.* Proteoliposomes containing PS3 cytochrome *cao* were prepared by the freeze–thaw–sonication procedure, and $H^+$-pumping activity was calculated from the pH change measured with a pH meter (Beckman 4500) and recorded with a strip-chart recorder (Sone & Yanagita, 1984).

*Spectrophotometric Assay.* Absorption spectra were measured with a recording spectrophotometer (Beckman DU70). The concentrations of cytochromes *cao* and *caa*$_3$ were determined by applying the value of $\Delta\epsilon_{mM}$(550–538 nm) = 21.3 of reduced cytochrome *caa*$_3$ (Sone & Yanagita, 1982) to cytochrome *cao*. The rebinding rate of CO to cytochrome *cao* after photolysis was determined as described previously (Sone et al., 1983).

*Resonance Raman Spectroscopy.* Raman scattering was excited at 406.7 nm with a $Kr^+$ ion laser (Spectra Physics, 2016) or at 441.6 nm with a He–Cd laser (Kimmon Electrics, CDR80MGH), and recorded on a JEOL 400D Raman spectrophotometer equipped with a cooled photomultiplier (Hamamatsu, R943-02) and a homemade computer-controller. A spinning cell (inner diameter = 3 mm, 1800 rpm) was used to avoid photodegradation of the sample. Chilled $N_2$ gas was flushed against the spinning cell to keep the sample temperature below 10 °C. Reduction of the enzyme was performed by the addition of a small excess amount of solid $Na_2S_2O_4$ under a $N_2$ atmosphere in the spinning cell. The carbon monoxide-bound enzyme was prepared by bubbling CO gas into the enzyme solution in the reduced state. Isotopically-labeled carbon monoxide ($^{13}C^{16}O$, 99%) was a product of ISOTEC Inc.

*Other Methods.* The oxidase activities for TMPD and cytochrome *c* were determined according to eq 1 (Nicholls & Sone, 1984) through measurements of pH changes upon addition of ascorbate (10 mM) as the final electron donor.

$$\text{ascorbate–H}^- + \text{H}^+ + {}^1/_2\text{O}_2 \rightarrow \text{dehydroascorbate} + \text{H}_2\text{O} \quad (1)$$

The net alkalization was determined by back-titration with 50 mM HCl. The molecular activity is expressed in terms of the turnover number of the enzyme, which is equal to twice the number of nanomoles of alkali formed per second per nanomoles of enzyme. Other procedures were the same as those described previously (Sone & Fujiwara, 1991). The activity measurements were carried out at 25 °C with a 2-mL cuvette containing 10 mM KCl, 10 mM sodium ascorbate, and 1 mM $NaP_i$ buffer (pH 6.95–7.0).

Recovery of the oxidase activity by dilution of cyanide-inhibited cytochrome *cao* was performed as follows. PS3 cytochrome *cao* (15 μM) was incubated with 0.5 mM KCN in 20 mM $NaP_i$ (pH 7.2) in the presence of 10 mM sodium ascorbate and 20 μM yeast cytochrome *c* at 20 °C for 40 min. An aliquot (10 μL) of this cyanocytochrome *cao* was then diluted into the reaction mixture (2 mL) as used for the activity measurements.

## RESULTS

*Oxidase Activity.* PS3 cytochrome *cao* actively oxidized yeast cytochrome *c* and TMPD. We reported previously that cytochrome *cao* oxidized cytochrome *c* with the same $K_m$ value but with a $v_{max}$ twice as large as that of cytochrome *caa*$_3$. The $1/v$ vs $1/[s]$ plot for the oxidation rate of TMPD as a substrate (s) gave a straight line, yielding $v_{max}$ values for cytochrome *cao* and *caa*$_3$ of 341 and 177 $s^{-1}$, respectively, while the $K_m$ value for TMPD was calculated to be 0.31 mM. Results with two other independent preparations of cytochrome *cao* were similar (not shown). These results suggest that heme replacement at the cytochrome *a*$_3$ site accelerates the internal electron transfer without changing the substrate-binding affinity.

*Proton Pumping Activity.* As reported previously, PS3 cytochrome *caa*$_3$ showed a high $H^+$-pumping activity (Sone & Hinkle, 1982; Sone & Yanagita, 1984). Figure 1 shows that cytochrome *cao* also pumps $H^+$. When a small amount of pH-adjusted yeast ferrocytochrome *c* was added as a reductant to liposomes containing cytochrome *cao*, the medium pH became more acidic, indicative of $H^+$ ejection. In the presence of an uncoupler, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP), this $H^+$ ejection was not observed, and a corresponding net alkalinization due to $H_2O$ formation took place.

The $H^+/e^-$ ratio, defined as the maximal number of protons ejected per molecule of ferrocytochrome *c* added, was determined to be 0.95 with PS3 cytochrome *cao*. This value was virtually the same as that (=1.05) of PS3 cytochrome *caa*$_3$ (Sone & Yanagita, 1984). Thus, it is likely that both cytochrome *caa*$_3$ and cytochrome *cao* work as a proton pump to translocate one proton upon electron transfer. However,

[1] Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; RR, resonance Raman; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone.

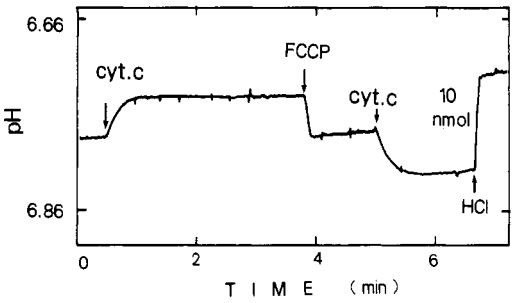


FIGURE 1: pH change due to proton pumping during cytochrome *c* oxidation by cytochrome *cao*. Proteoliposomes containing 13 μg of cytochrome *cao* and 4 mg of soybean phospholipids in 0.1 mL were suspended in 2 mL of reaction medium composed of 25 mM $K_2SO_4$, 2.5 mM $MgSO_4$, 0.1 mM 4-morpholinepropanesulfonic acid–KOH buffer, and 100 ng/mL valinomycin at 38 °C. The arrows mean the addition of ferrocytochrome *c* (4.1 nmol), FCCP (100 ng), and HCl (10 nmol).

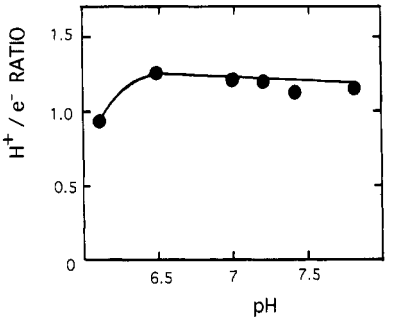


FIGURE 2: pH dependence of the $H^+/e^-$ ratio determined by the initial rate method. The proteoliposomes containing cytochrome *cao* and the reaction medium used were the same as those used in Figure 1 except that 0.75 mM 4-morpholinepropanesulfonic acid–KOH buffer adjusted at the indicated pH value was used and reduced cytochrome *c* was regenerated in the presence of 5 mM sodium ascorbate. The $H^+/e^-$ ratio was calculated according to the following formula (Sone & Yanagita, 1984): $H^+/e^- = v_1/2v_2 - 0.5$, where $v_1$ is the initial rate of $H^+$ ejection and $v_2$ is the steady-state net alkalinization rate in the presence of FCCP (1 μg).

the true $H^+/e^-$ ratio of these cytochrome *c* oxidases might be slightly higher than unity, because even 30 s after the addition of ferrocytochrome *c*, when the pH change was maximal, cytochrome *c* was not oxidized completely.

Figure 2 shows the effects of pH on the $H^+/e^-$ ratio which was determined by the initial rate method; the initial rate of pH change due to ferrocytochrome *c* oxidation was compared with the rate of $H^+$ uptake in the presence of FCCP. The $H^+/e^-$ ratios thus obtained were 1.14–1.27 around pH 6.5–7.8 as shown in Figure 2. These values were again very close to those of cytochrome $caa_3$, which were about 1.3 at this pH range (Sone & Yanagita, 1984). Slightly smaller values for cytochrome *cao* than for cytochrome $caa_3$ may not be due to heme replacement, since different preparations of cytochrome $caa_3$ gave values similar to those in Figure 2. It is thus concluded that the substitution of heme O for heme A scarcely affects the proton pumping activity of PS3 cytochrome oxidase.

*Liganding Effect of Cyanide.* Cyanide is known to bind to the binuclear center, including the spin-state conversion and inhibiting the oxidase activity (Nicholls et al., 1972; Nicholls & Chance, 1974). We examined the effects of cyanide binding on the visible absorption spectra of PS3 cytochrome *cao* and analyzed the inhibition kinetics in comparison with the effects on cytochrome $caa_3$ (Sone & Nicholls, 1985). Figure 3 shows the effect of cyanide on the spectra of PS3 cytochrome *cao* in the mixed-valence and fully reduced forms. The mixed-valence cyanide form (B) shows a spectrum very similar to

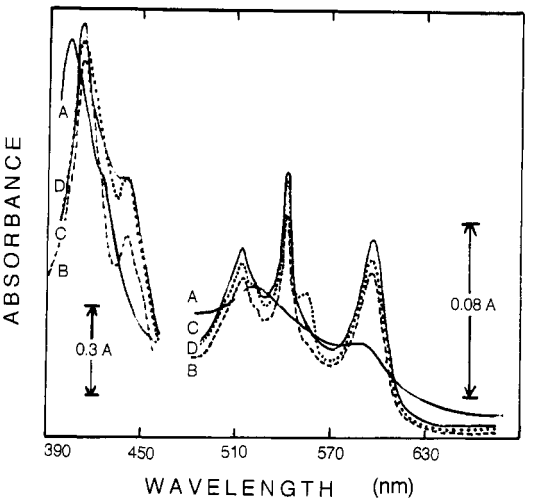


FIGURE 3: Absorption spectra of cytochrome *cao* and its cyanide complex. (A) Solid line: oxidized form (3 nmol) in a 100-μL aliquot composed of 50 mM $NaP_i$ (pH 7.5) and 0.5% Tween 80. (B) Dashed line: cyanide-bound mixed-valence form, prepared from (A) by incubation with 1 mM KCN in the presence of 5 mM ascorbate and 0.05 mM TMPD. (C) Solid line: fully-reduced form prepared from (A) as in (B) although KCN was replaced by a few grains of $Na_2S_2O_4$. (D) Dotted line: fully-reduced and cyanide-bound form prepared from (C) through incubation for 10 min in the presence of 5 mM KCN supplemented with a grain of $Na_2S_2O_4$.

that of the fully reduced form (C), indicating that the contribution of the high-spin ferrous and ferric forms of cytochrome *o* to the α-band region is small but the difference between the fully reduced form (C) and the cyanide-bound fully reduced form (D) is evident at around 560 nm. Conversion of the spin state from high- to low-spin due to cyanide binding to ferrous hemes caused the appearance of a new peak around 560 nm, while in the case of cytochrome $caa_3$ a new peak appeared around 590 nm due to low-spin heme A (Sone & Nicholls, 1985). These spectral changes upon KCN addition clearly indicate that the cytochrome responsible for the spectral change is cytochrome *o* but is not cytochrome $a_3$.

Figure 4 illustrates aspects of the spectral change upon development of the ferric cyanide complex of cytochrome *cao*. As was observed with the mammalian enzyme (Nicholls et al., 1972) and PS3 cytochrome $caa_3$ (Sone & Nicholls, 1985), a single cyanide-bound compound is formed with a single set of isosbestic points (414, 467, 533, and 572 nm), although their individual wavelengths are different from those of cytochrome $caa_3$ due to the difference of the heme. Shifts of these isosbestic points toward shorter wavelengths as well as the peak at 421 nm and the trough at 403 nm indicate that the cyanide-bound heme is heme O but not heme A. When the logarithm of the absorbance change is plotted against time as shown by the inset of Figure 4, it becomes clear that cytochrome *cao* binds cyanide biphasically, *ca.*, 50% of the change occurring rapidly and the remaining 50% taking place with a half-time of 30 min. It is also noteworthy that a shoulder around 448 nm is found only in the later stage of inhibition, suggesting that the spectral change is due to the formation of $E(CN^-)'$ (cf. eq 2 under Discussion).

*Kinetics of Cyanide Binding.* The catalytic activity of cytochrome *cao* was inhibited slowly upon the addition of a small amount of cyanide, as in the case of the mammalian enzyme (Nicholls et al., 1972) and PS3 cytochrome $caa_3$ (Sone & Nicholls, 1985). Figure 5 shows the progressive inhibition of oxidase activity after the addition of cyanide, plotted logarithmically. This process is also biphasic. As indicated

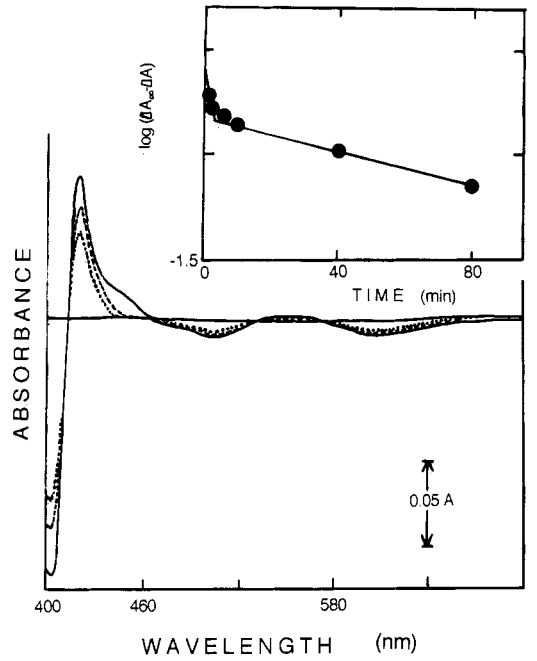


FIGURE 4: Time course of cyanide binding to oxidized PS3 cytochrome *cao*. Difference spectra of cyanide-bound oxidized enzyme minus oxidized enzyme were taken as follows: solid line, without cyanide; dotted line, 1.5 min after addition of KCN; dashed line, after 20 min; solid line, after 80 min. PS3 cytochrome *cao* (6 μM) and 1.25 mM KCN were used at 20 °C. The inset shows the kinetics of cyanide binding. Differences between absorbances at 421 and 403 nm are plotted against time.

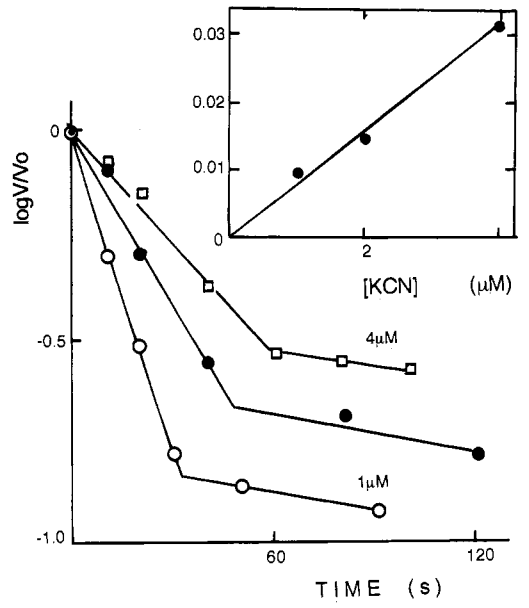


FIGURE 5: Kinetics of cyanide inhibition. The logarithms of the residual oxidase activity are plotted against the time after addition of KCN. The activity of PS3 cytochrome oxidase (120 pmol) was monitored with a pH meter as described under Materials and Methods using 20 μM yeast cytochrome *c*. The inset plots the relationship between [KCN] and the initial apparent first-order rate constant.

in the inset, the rapid inhibition occurs at a rate proportional to the cyanide concentration, with a rate constant of $8 \times 10^3$ $M^{-1}$ $s^{-1}$, which is slightly faster than those of mammalian cytochrome *c* oxidases and PS3 cytochrome $caa_3$. The slower phase is not dependent on cyanide concentration, and thus may reflect an intramolecular conformational change (cf. eq 2).

When the reciprocals of the remaining activities of the initial and final phases of cyanide inhibition were plotted against

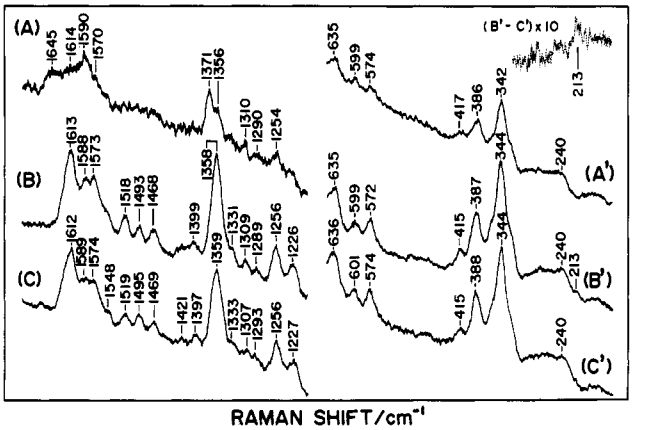


FIGURE 6: Resonance Raman spectra of PS3 cytochrome *cao* excited at 441.6 nm. (A and A′) Resting state; (B and B′) fully-reduced state; (C and C′) CO-bound fully-reduced state. The dotted line shows the Raman difference spectrum (spectrum B′ – spectrum C′).

KCN concentrations (Dixon plot), it gave straight lines depending on the concentrations of cytochrome *c* (figure not shown). The apparent $K_i$ values thus obtained were smaller than those of cytochrome $caa_3$: 5 and 1.6 μM, respectively (Sone & Nicholls, 1985). Since $k_{on}$ was almost the same in these three enzymes, the difference of $K_i$ should be due to the rate of dissociation of cyanide.

When the cyanide-inhibited cytochrome *cao* was diluted by buffer, the oxidase activity was restored. The oxidase activities at a given time ($V_t$) after the start of dilution and at long times after that ($V_0$) were determined in a way similar to those used for Figure 5. When $\log[(V_0 - V_t)/V_0]$ was plotted against time, it gave a straight line and its slope yielded a dissociation rate constant ($k_{off}$) of $4.8 \times 10^{-3}$ $s^{-1}$ (figure not shown). This value is slightly smaller than that of PS3 cytochrome $caa_3$, but similar to that of mammalian cytochrome $aa_3$, indicating that the smaller $K_i$ for cytochrome *cao* than for cytochrome $caa_3$ is mainly due to the smaller dissociation rate constant of the former.

*CO Binding to Cytochrome cao.* CO is known to bind to the binuclear center similar to $O_2$. The CO difference spectrum (reduced CO-bound minus reduced) of PS3 cytochrome *cao* gave positive peaks at 567, 532, and 417 nm and a trough at 432 nm (Sone & Fujiwara, 1991). The difference spectrum was very close to that of *E. coli* cytochrome *bo* (Kita et al., 1984; Matsushita et al., 1984), but different from those of PS3 cytochrome $caa_3$ (Sone & Yanagita, 1982) and a mammalian cytochrome $aa_3$, indicating that the heme species responsible for CO binding is the same as that in cytochrome *bo* despite the fact that the compositions of hemes in a whole molecule are dissimilar. The rate constant of CO rebinding to cytochrome *cao* after flash photolysis was 26–29 $s^{-1}$ at 25 °C, almost the same as that (28–30 $s^{-1}$) of PS3 cytochrome $caa_3$ (Sone et al., 1983). Accordingly, the replacement of heme from heme A to heme O at the binuclear center barely affects the rate of CO binding.

*Resonance Raman Spectroscopy.* Figure 6 shows resonance Raman (RR) spectra of cytochrome *cao* in the higher (1200–1700 $cm^{-1}$; spectra A, B, and C) and lower frequency regions (150–650 $cm^{-1}$; spectra A′, B′, and C′) excited at 441.6 nm. Spectra (A, A′), (B, B′), and (C, C′) represent the resting, reduced, and reduced CO-bound forms of cytochrome *cao*, respectively. The $\nu_4$ band (Abe et al., 1978), which appears at 1371 $cm^{-1}$ for the resting state (spectrum A), indicates the presence of ferric hemes. The shoulder at 1356 $cm^{-1}$ is assignable to a ferrous heme and suggests that a small amount

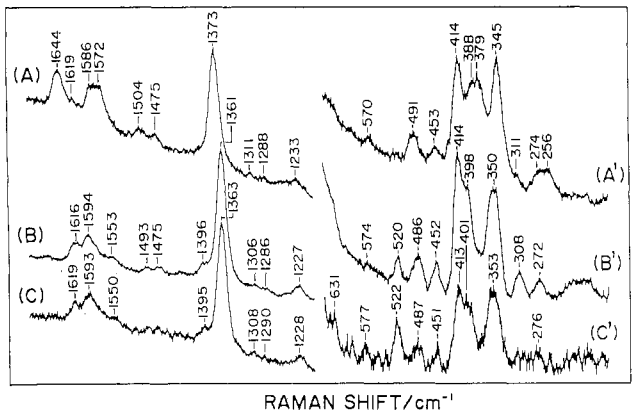


FIGURE 7: Resonance Raman spectra of PS3 cytochrome *cao* excited at 406.7 nm. (A and A′) Resting state; (B and B′) fully-reduced state; (C and C′) CO-bound fully-reduced state.

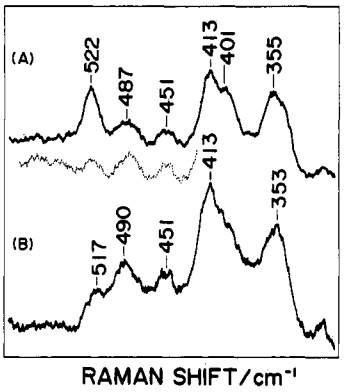


FIGURE 8: Resonance Raman spectra of CO-bound fully-reduced cytochrome *cao* excited at 406.7 nm. (A) $^{12}C^{16}O$; (B) $^{13}C^{16}O$. The dotted curve shows the spectrum obtained for $^{12}C^{16}O$-bound enzyme when the spinning of the cell was stopped.

of the cytochrome was photoreduced as was noted for cytochrome $aa_3$ (Adar & Yonetani, 1978; Ogura et al., 1985). On the contrary, the formyl stretching mode ($\nu_{CH=O}$), which is clearly observed around 1665 $cm^{-1}$ for the reduced $aa_3$-type cytochromes and is assigned to cytochrome $a_3$ (Babcock, 1988), was not observed for the reduced state (spectrum B). For the fully-reduced species, the $\nu_4$ band appeared at 1358 $cm^{-1}$ (spectrum B). There is a small shoulder at 213 $cm^{-1}$ which is absent in spectra A′ and C′. The dotted curve at the top represents the difference spectrum obtained by subtracting spectrum C′ from spectrum B′. This clearly shows the presence of a band at 213 $cm^{-1}$ for the reduced state. This band is assigned to the Fe–His stretching mode ($\nu_{Fe-His}$). Spectra B and B′ are virtually identical with those of $aa_3$-type cytochromes except for the lack of the $\nu_{CH=O}$ band and the low intensity of the $\nu_{Fe-His}$ band (Ogura et al., 1984). We see no significant difference between spectra B and C except for the presence of a shoulder on the higher frequency side of the 1359-$cm^{-1}$ band, which indicates an upshift of the $\nu_4$ mode for cytochrome $o^{2+}$–CO. These facts mean that most of the observed bands in spectra B and C arise from cytochrome *c* and cytochrome *a* moieties.

Figure 7 shows the 406.7-nm-excited RR spectra of PS3 cytochrome *cao* in the higher (1200–1700 $cm^{-1}$; spectra A, B, and C) and lower (150–650 $cm^{-1}$; spectra A′, B′, and C′) frequency regions, respectively. The band at 1644 $cm^{-1}$ may contain contributions from the $\nu_{10}$ mode of ferric low-spin heme and the $\nu_{CH=O}$ mode of cytochrome *a*. The spectral pattern in the lower frequency region is apparently distinct from that of $aa_3$-type cytochrome oxidases. This is, however, due to a larger contribution of the cytochrome *c* moiety to the Raman spectra upon excitation at 406.7 nm, since the absorption maxima of cytochrome *c*, which are located at 407 and 416 nm for the oxidized and reduced states, respectively, are noticeably close to the Raman excitation wavelength. The RR spectra of the CO-bound form (spectra C and C′) resemble those of the fully-reduced form except for intensification of the band at 522 $cm^{-1}$. Since the band at 522 $cm^{-1}$ may be due to the Fe–CO stretching ($\nu_{Fe-CO}$) mode, we examined the CO isotope effect on the band.

Figure 8 shows the RR spectra in a low-frequency region for $^{12}C^{16}O$-bound (A) and $^{13}C^{16}O$-bound reduced enzymes (B). It is clear that the band at 522 $cm^{-1}$ for $^{12}C^{16}O$ exhibits a downshift to 517 $cm^{-1}$ for $^{13}C^{16}O$. The dotted curve displays the spectrum obtained when the spinning of the cell for $^{12}C^{16}O$-bound enzyme was stopped. Intensity reduction of the band at 522 $cm^{-1}$ is evident, indicating that the stop of spinning of the Raman cell induced photodissociation of CO. The band at 487 $cm^{-1}$ exhibits no intensity reduction upon stop of spinning of the cell, and furthermore, it is not shifted with $^{13}C^{16}O$. Therefore, it cannot be assigned to $\nu_{Fe-CO}$, although the presence of two $\nu_{Fe-CO}$ RR bands at 523 and 489 $cm^{-1}$ was pointed out for cytochrome *bo* (Uno et al., 1985).

## DISCUSSION

We found that PS3 cytochrome *cao* could oxidize cytochrome *c* and TMPD with the same $K_m$ with concomitant vectorial proton translocation across the membrane as PS3 cytochrome $caa_3$ does. The efficiency of proton translocation ($H^+/e^-$ ratio) was nearly constant (about 1.2) in the pH range from 6.5 to 7.8. The similar pH dependence of the $H^+/e^-$ ratio was also observed with cytochrome $caa_3$. Therefore, the effect of substitution of heme O for heme A is quite small. It was spectroscopically confirmed that heme replacement really does take place at the cytochrome $a_3$ site, since RR spectra showed the absence of the $\nu_{CH=O}$ band of cytochrome $a_3$ for cytochrome *cao*, and upon addition of cyanide the visible absorption spectra exhibited changes corresponding to the spin-state conversion of heme O for cytochrome *cao* but that of heme A for cytochrome $caa_3$. The heme replacement causes some quantitative changes. The molecular activity of cytochrome *cao* was as twice high as that of cytochrome $caa_3$, and the $K_i$ value for cyanide was lower with cytochrome *cao* than with cytochrome $caa_3$.

Kinetic constants for the formation of cyanide complex and its dissociation were measured for cytochrome *cao* (Figures 3–5). Table 1 compares these parameters with the corresponding values of PS3 cytochrome $caa_3$ and beef heart cytochrome $aa_3$. The parameters were obtained with the assumption that the binding of cyanide to the enzyme proceeds in two steps (Nicholls et al., 1972):

$$E + CN^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E(CN^-)' \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\text{–}CN^- \qquad (2)$$

where $E(CN^-)'$ is a putative intermediate. When $k_2 > k_{-2}$ and neither is rapid, the overall (final) inhibition constant $K_i$ is equal to $k_{-1}k_{-2}/k_1k_2$. The intermediate species, $E(CN^-)'$, is then responsible for the initial inhibition with the constant $K_\alpha$ [$=(k_{-1} + k_2)/k_1$]. The initial binding rate ($k_{on}$) is controlled by $k_1$, and the initial dissociation rate ($k_{off}$) is equal to $k_{-1}k_{-2}/(k_{-1} + k_2)$. $K_i$ for cyanide was much smaller with PS3 cytochrome *cao* than that with PS3 cytochrome $caa_3$, but the former was not so small as that with mammalian cytochrome $caa_3$. The difference in $K_i$ between cytochrome $caa_3$ and cytochrome *cao* was attributable to the difference in $k_{off}$ being

Table 1: Comparison of the Rate and Equilibrium Constants Obtained for the Reaction of Cyanide with PS3 Cytochrome *cao*, PS3 Cytochrome $caa_3$, and Mammalian (Beef Heart) Cytochrome $caa_3$[a]

| obsd constant | equiv kinetic expressioin | values | | |
|---|---|---|---|---|
| | | PS3 cyt *cao* | PS3 cyt $caa_3$ | mammalian cyt $caa_3$ |
| $K_i$ (M) | $k_{-1}k_{-2}/k_1k_2$ | $0.4 \times 10^{-6}$ | $1.6 \times 10^{-6}$ | $0.1 \times 10^{-6}$ |
| $K$ (M) | $(k_{-1} + k_2)/k_1$ | $1.2 \times 10^{-6}$ | $5.0 \times 10^{-6}$ | $0.8 \times 10^{-6}$ |
| $k_{on}$ ($M^{-1}$ $s^{-1}$) | $k_1k_2/(k_{-1} + k_2)$ | 8000 | 6700 | 4300 |
| $k_{off}$ ($s^{-1}$) | $k_{-1}k_{-2}/(k_{-1} + k_2)$ | $4.8 \times 10^{-3}$ | $10.8 \times 10^{-3}$ | $4.3 \times 10^{-3}$ |
| $Kk_{off}/K_i$ ($s^{-1}$) | $k_2$ | $1.4 \times 10^{-2}$ | $3.4 \times 10^{-2}$ | $1.4 \times 10^{-2}$ |
| $k_{off}/k_{on}$ (M) | $k_{-1}k_{-2}/k_1k_2$ | $0.6 \times 10^{-6}$ | $1.6 \times 10^{-6}$ | $0.46 \times 10^{-6}$ |

[a] Data for PS3 cytochrome $caa_3$ (Sone & Nicholls, 1985) and for beef heart cytochrome (Nicholls et al., 1972).

much larger for PS3 cytochrome $caa_3$ than for PS3 cytochrome *cao* (Sone & Nicholls, 1985). The ratio of $k_{off}/k_{on}$ should be identical with $K_i$, as required by eq 2. This is almost satisfied with PS3 cytochrome *cao* as with PS3 cytochrome $caa_3$, but not with mammalian cytochrome $caa_3$, indicating that a more complicated mechanism is necessary.

There are a few reports on RR spectra of cytochrome *o* (Uno et al., 1985; Wang et al., 1993; Yumoto et al., 1993). Uno et al. (1985) pointed out the presence of two $\nu_{Fe-CO}$ RR bands for *E. coli* cytochrome *bo*, but Wang et al. (1993) stressed a single $\nu_{Fe-CO}$ RR band and the similarity of its frequency to that of cytochrome $aa_3$. Yumoto et al. (1993) measured RR spectra of cytochrome *aco* isolated from a facultative alkalophilic *Bacillus* and found that cytochrome *o* stays in the five-coordinate high-spin state and its $\nu_{Fe-CO}$ RR band is located at 520 $cm^{-1}$, similar to cytochrome $aa_3$. In the present study, the RR spectra of cytochrome *cao* were found to bear close resemblance to those of PS3 cytochrome $caa_3$ (Ogura et al., 1984; Sone et al., 1986) with two exceptions: first, the $\nu_{CH=O}$ band due to cytochrome $a_3$ was not seen in cytochrome *cao*; and second, the intensity of the $\nu_{Fe-His}$ band at 213 $cm^{-1}$ was reduced. The lack of the $\nu_{CH=O}$ band is consistent with the replacement of cytochrome $a_3$ by cytochrome *o* whose prosthetic group (heme O) has no formyl side chain (Puustinen et al., 1991; Wu et al., 1992). The lower intensity of the 213-$cm^{-1}$ band does not mean that the stoichiometry of cytochrome *o* in the molecule is low, but is probably due to poorer resonance effects for ferrocytochrome *o* ($\lambda_{max}$ = 430 nm) compared with ferrocytochrome $a_3$ ($\lambda_{max}$ = 443 nm) upon excitation at 441.6 nm. The $\nu_{Fe-His}$ frequencies have been found at 220–221 $cm^{-1}$ for hemoglobins in the R state (Nagai et al., 1980) and myoglobins (Kitagawa et al., 1979), 210–214 $cm^{-1}$ for mammalian cytochrome $a_3$ (Ogura et al., 1983), 215–216 $cm^{-1}$ for hemoglobins in the T state (Nagai et al., 1980), and 240–250 $cm^{-1}$ for peroxidases (Teraoka et al., 1981, 1983; Hashimoto et al., 1986). In this context, the frequency at 213 $cm^{-1}$ for PS3 cytochrome *cao* is consistent with its being a terminal oxidase.

The band at 522 $cm^{-1}$ of CO-bound cytochrome *cao* was photosensitive and exhibited a downshift to 517 $cm^{-1}$ upon $^{13}C^{16}O$ substitution. Therefore, this band was assigned to the $\nu_{Fe-CO}$ mode. Since the band at 487 $cm^{-1}$ in Figure 8 did not exhibit a $^{13}C^{16}O$ isotopic frequency shift, this band cannot be ascribed to the $\nu_{Fe-CO}$ mode, in agreement with Wang et al. (1993). It is known that hemoglobin and myoglobin give the $\nu_{Fe-CO}$ band at around 507 $cm^{-1}$ (Tsubaki et al., 1982), while cytochrome $a_3$ gives it at 516–520 $cm^{-1}$ (Argade et al., 1984; Hirota et al., 1993). The $\nu_{Fe-CO}$ frequency of cytochrome *cao* suggests that the structural characteristics in the heme environments of cytochrome *o* are rather similar to those of cytochrome $a_3$ than those of hemoglobin and peroxidases, although heme A at the binuclear center is replaced by heme O in PS3 cytochrome *cao*. The similar feature is pointed out for *E. coli* cytochrome *bo* (Wang et al., 1993). It is also noteworthy that the recombination rate constant of CO to PS3 cytochrome *cao* was very close to that of PS3 cytochrome $caa_3$ (Sone et al., 1983).

Since the absorption maximum of reduced cytochrome *a* is located at 443 cm, it is expected that the contribution of cytochrome *a* to the RR spectra upon excitation at 441.6 nm is large (Figure 6, spectra B and C). The close similarity of these spectra to the corresponding spectra of PS3 cytochrome $caa_3$ suggests that there is no significant structural difference in the cytochrome *a* moiety between cytochromes *cao* and $caa_3$. The RR spectra excited at 406.7 nm are considered to contain a dominant contribution from the cytochrome *c* moiety, since the absorption maxima of oxidized and reduced cytochrome *c* are located at 407 and 416 nm. In fact, the spectra shown in Figure 7 bore close resemblance to those of horse heart cytochrome *c*, particularly in the lower frequency region (Hildebrandt et al., 1990). The cytochrome *c* moiety in PS3 cytochrome $caa_3$ as well as in cytochrome *cao* became a part of subunit II due to gene fusion, and its amino acid sequence deduced from the DNA sequence is a little different from those of mammalian cytochrome *c* (Ishizuka et al., 1990).

In conclusion, PS3 cytochrome *cao* is a proton pump and shows reaction kinetics and cyanide inhibition patterns which are very similar to those of cytochrome $caa_3$ except for slight differences in the magnitude of the rate constants. Their RR spectra indicate that the protein structures in the heme vicinity of cytochrome *c*, as judged from the $\nu_{Fe-His}$ and $\nu_{Fe-CO}$ frequencies, are very close to those of cytochrome $a_3$ but are distinct from those of hemoglobin and peroxidases.

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