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# Carbon Monoxide Complex of Cytochrome $b_5$ at Acidic pH

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**CO complex of cyt $b_5$ generated at acidic pH is investigated by absorption, resonance Raman (RR), and far UV CD measurements. The Soret maximum wavelength blue-shifted to 420 nm with other absorption bands observed around 540 and 570 nm for reduced cyt $b_5$ upon interaction with CO at acidic pH (pH 3.1–3.5). Under this condition, the iron–carbon stretching RR band was observed at 529 $cm^{-1}$ (520 $cm^{-1}$ for $C^{18}O$), which indicated formation of a heme—CO adduct with a histidine as an axial ligand. Heme dissociated from the reduced cyt $b_5$ protein at pH ~3.5, whereas its rate decreased under CO atmosphere compared with $N_2$ atmosphere, due to formation of a heme—CO adduct with a histidine as an axial ligand.** © 2001 Academic Press

Cytochrome $b_5$ (cyt $b_5$) is often bound to the microsome membrane and functions as a member of the microsome electron transport system. Microsomal cyt $b_5$ consists of a hydrophilic domain and another hydrophobic one (1). The hydrophilic domain is involved in electron transfer of proteins (2), whereas the hydrophobic domain is responsible for the anchoring of the protein to the membrane (3). The hydrophilic domain is consisted with about 100 amino acid residues and contains a noncovalently bound heme, which is coordinated by two histidines, His39 and His63 (4, 5). Cyt $b_5$ can be proteolyzed by trypsin to produce a soluble N-terminal fragment consisting of 84 residues which is termed $Tb_5$, whereas lipase can proteolyze the protein to produce a 93-residue fragment referred to as $Lb_5$ (6). The stability of cyt $b_5$ has been studied by heat (7–9) and denaturants (9). For example, the heme of cyt $b_5$ has been shown to dissociate from the protein by denaturing the protein with urea, and the denaturant concentration and temperature for dissociation of the heme have been used to estimate the protein stability (9).

Carbon monoxide (CO) binds to various heme proteins and has been used to study their structure–function relationships (10–14). The iron–carbon stretching ($\nu_{Fe\text{-}C}$) frequencies obtained by resonance Raman (RR) spectroscopy exhibit a negative linear correlation with the IR carbon–oxygen ($\nu_{CO}$) stretching frequencies for a variety of heme—CO adducts (10–14), and the electrostatic field near the bound CO has been indicated to exert a greater influence on the polarization of CO and its Fe—C and C—O frequencies than does steric hindrance (15–18). Cytochrome P450, *b*-type cytochromes with a cysteine coordinated to the heme iron, is stabilized by formation of a CO complex (19). For cyt *c*, the heme is coordinated with a histidine and a methionine in its native folded state, while CO was shown to bind to the heme instead of methionine when it was unfolded with a high amount of guadinium hydrochloride (Gdn · HCl) (20). This CO binding property has been utilized to study its folding character (20). To examine the protein structural properties of cyt $b_5$ in detail, we studied the interaction of cyt $b_5$ with CO at acidic pH (pH 3.1–3.5). We show that CO, a neutral ligand, can compete with the histidine ligand for coordination to the heme iron at acidic pH and would decrease the rate of heme dissociation from the protein. This is to our knowledge the first detailed study on the interaction of cyt $b_5$ with CO.

## MATERIALS AND METHODS

*Protein purification.* The pUC19 plasmid containing the synthetic gene encoding the trypsin-solubilized bovine liver microsomal cytochrome $Tb_5$ was a generous gift from Professor A. G. Mauk, and the protein was purified as reported (21). Lyophilized cyt $b_5$ was dissolved before each experiment, and its concentration was adjusted

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by the Soret absorption maximum of the ferric form ($\epsilon_{414}$ = 117,000 $M^{-1}cm^{-1}$) (22).

*Spectroscopic measurements.* Absorption spectra of cyt $b_5$ in the presence and absence of CO at acidic pH were measured at 15°C on a Shimadzu UV-3100PC spectrophotometer. A tightly sealed quartz cell with cyt $b_5$ dissolved in 10 mM sodium phosphate buffer, pH 7.4, was filled with $N_2$ using a vacuum line. The sample solution was then reduced with dithionite (final concentration, 0.5 mM), added with 20% acetic acid under $N_2$ atmosphere, and kept under $N_2$ atmosphere or refilled with CO. Time courses of the absorption changes of reduced cyt $b_5$ incubated with $N_2$ and CO at acidic pH were monitored at 423 and 420 nm, respectively.

RR scattering was excited at 406.7 nm with a $Kr^+$ ion laser (Spectra Physics, 2060) and detected with a CCD (Astromed CCD, 3200) attached to a single polychromator (Ritsu Oyo Kogaku, DG-1000). The slit width and slit height were set to be 200 $\mu$m and 10 mm, respectively. The excitation laser beam power (at the sample point) was adjusted to 10 mW. RR measurements were carried out at room temperature with a spinning cell (3000 rpm). The data accumulation time was 400 s. Raman shifts were calibrated with acetone and toluene, and the accuracy of the peak positions of the Raman bands was $\pm$ 1 $cm^{-1}$. Cyt $b_5$ dissolved in 10 mM sodium phosphate buffer, pH 7.4, was added with 0.1 M HCl to make the solution acidic (pH 3.1). The quartz cell containing the sample solution was filled with $N_2$, added with dithionite (final concentration, 1 mM), and finally refilled with $C^{16}O$ or $C^{18}O$ (Isotec, 98 atom % for $^{18}O$).

Circular dichroism (CD) ellipticity and spectra were measured at 15°C on a Jasco J-720 spectropolarimeter in a quartz cell with a path length of 1 cm. Cyt $b_5$ solution with 10 mM sodium phosphate buffer, pH 7.4, was added with dithionite (final concentration 0.12 mM) under $N_2$ or CO atmosphere to measure the reduced cyt $b_5$ spectra. The pH value of the sample solution was then decreased to 3.3 with 0.1 M HCl under $N_2$ or CO atmosphere, and the CD ellipticity change was monitored at 220 nm. The CD spectra for unfolded cyt $b_5$ were measured after 10-h incubation at pH 3.3.

## RESULTS AND DISCUSSION

*Effect of CO binding on absorption spectra of reduced cyt $b_5$.* The Soret absorption band of reduced cyt $b_5$ at acidic pH (pH ~3.5) exhibited its maximum intensity at 423 nm (Fig. 1, a), which intensity gradually decreased due to dissociation of the heme from the denatured protein. Addition of CO to reduced cyt $b_5$ at acidic pH caused the maximum wavelength of the Soret band gradually shift to 420 nm (Fig. 1, b). In addition to this blue-shift of the Soret band, the $\alpha$ and $\beta$ absorption bands were detected at about 540 and 570 nm. Under CO atmosphere at acidic pH, the absorption spectrum of reduced cyt $b_5$ therefore corresponded very well with that of CO-bound myoglobin, which suggested dissociation of one of the histidine ligands from the heme iron and formation of a heme—CO adduct with the other histidine as an axial ligand for reduced cyt $b_5$ upon interaction with CO. Since the wavelength shift of the Soret band occurred relatively slowly and completed in about 40 min (data not shown), dissociation of one of the histidine ligands from the heme should take place very slowly. However, no change was observed in the absorption spectra of reduced cyt $b_5$ at neutral pH or oxidized cyt $b_5$ at acidic pH by introducing CO.

Although a protonated histidine was uncoordinated to the heme iron due to CO coordination, at least one of the histidines should remain coordinated to the heme to prevent dissociation of the heme from the protein. Since it was demonstrated by mutant studies that the fluctuating loop including His63 (23, 24) possesses a significantly slower heme dissociation rate and a faster heme association rate than the other loop including His39 (25), His63 is suggested to remain coordinated and His39 to dissociate from the heme when CO binds to reduced cyt $b_5$ at acidic pH.

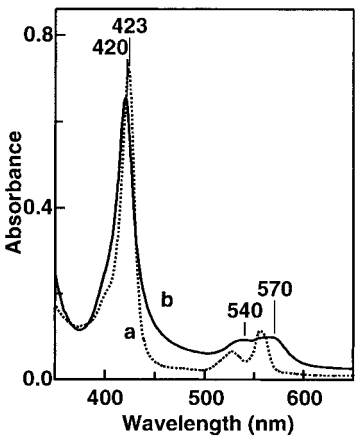


**FIG. 1.** Absorption spectra of reduced cyt $b_5$ (7 $\mu$M) at pH 3.53 under $N_2$ (a, dotted line) and CO (b, solid line) atmospheres. Spectrum b was measured after 40-min incubation with CO. Measured at 15°C.

*RR spectra of CO-bound cyt $b_5$.* RR spectroscopy is a powerful method to investigate the Fe—C bond character in heme proteins. The Raman band at 529 $cm^{-1}$ for reduced cyt $b_5$ at pH 3.1 under $C^{16}O$ atmosphere shifted to a lower frequency at 520 $cm^{-1}$ with $C^{18}O$, and the difference spectrum demonstrates the frequency shift without affecting other heme related bands significantly (Fig. 2). The observed isotopic wavenumber shift of 9 $cm^{-1}$ is in good agreement with the calculated value for an iron–carbon two-atom model. This band is therefore assigned to the $\nu_{Fe\text{-}C}$ mode, which provides direct evidence of CO coordination to the heme iron of reduced cyt $b_5$ at acidic pH.

The $\nu_{Fe\text{-}C}$ and $\nu_{CO}$ frequencies of CO-bound heme proteins reflect the electronic structure of the heme and its environment (10, 18) The axial ligand and electrostatic fields of heme proteins have been shown to influence significantly the $\nu_{Fe\text{-}C}$ and $\nu_{CO}$ frequencies of the bound CO (15, 16, 26, 27). For example, existence of positive charges from proton donating residues close to the bound CO causes the electron density of the $\pi^*$ orbital of the bound CO to increase through the $\pi$ back-bonding from the filled *d* orbital of the iron, which causes increase in the $\nu_{Fe\text{-}C}$ frequency. The $\nu_{Fe\text{-}C}$ frequency at 529 $cm^{-1}$ for CO-bound cyt $b_5$ was within the frequency region of the $\nu_{Fe\text{-}C}$ frequencies for histidine-coordinated heme species, which indicated formation of

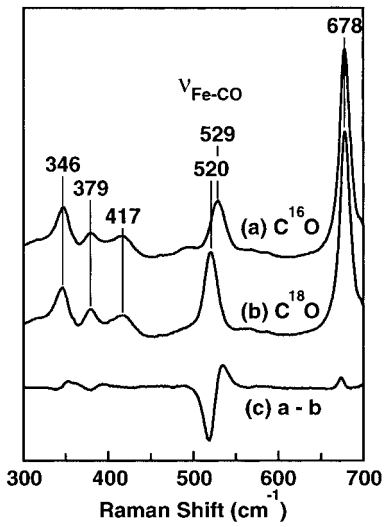


**FIG. 2.** RR spectra in the 300–700 $cm^{-1}$ region for $C^{16}O$ (a) and $C^{18}O$ (b) adducts of reduced cyt $b_5$ (25 $\mu$M) at pH 3.1 and their difference spectrum, a–b (c). The ordinate scales of spectra a and b are normalized by the intensity of porphyrin bands. Measured at room temperature.

a 6-coordinate heme—CO adduct with a histidine as an axial ligand. The frequency, however, was relatively high, which suggested existence of positive charges around the bound CO. The positive charges could be attributed to a protonated histidine sited nearby the bound CO and/or to the unfolding of the protein under acidic condition. Actually, the protein was mostly unfolded for CO-bound cyt $b_5$ at acidic pH (see section 3.4).

*Heme dissociation at acidic pH.* The intensity of the Soret absorption band of reduced cyt $b_5$ decreased gradually at pH 3.46 under $N_2$ and CO atmospheres (Fig. 3). The rate of the absorption change was faster for lower pH and very sensitive to the pH value. Although the overall time courses looked unusual, the rate constants of the absorption change at the early stage were $0.11 \pm 0.1$ and $0.064 \pm 0.02$ $min^{-1}$ under $N_2$ and CO atmospheres, respectively. The absorption change at the early stage could be due to dissociation of one of the coordinated histidines from the heme iron with the other histidine remaining coordinated, whereas the slower absorption change could be attributed to dissociation of the other histidine from the heme and thus the heme from the protein. According to the absorption spectrum, CO bound to the heme within 40 min at acidic pH (data not shown), which was slower than dissociation of the first histidine from the heme iron but faster than dissociation of the second histidine (Fig. 3b). These results indicated that CO therefore binds after dissociation of the first histidine from the heme iron but before dissociation of the second histidine.

The rate of heme dissociation from reduced cyt $b_5$ at acidic pH was slower under CO atmosphere than under $N_2$ atmosphere. The decrease in the heme dissociation rate by CO incubation was also detected by RR spectroscopy (data not shown). The decrease in the dissociation rate could be due to inhibition of dissociation of the coordinated histidine by formation of a His—Fe(II)—CO species, which has a stronger ligand field than His—Fe(II)—His, especially in the acidic pH region.

*CD spectra of cyt $b_5$ at acidic pH.* The CD spectra of reduced cyt $b_5$ at neutral pH under $N_2$ and CO atmospheres exhibited very similar protein folding character. The unfolded spectra of reduced cyt $b_5$ measured after incubation for 10 h at pH 3.3 under $N_2$ and CO atmospheres were also very similar between each other. The time course of the CD ellipticity at 220 nm after adjusting the sample solution to acidic pH (3.3) was monitored to elucidate the changes in the $\alpha$-helical content of the protein and its structure. The amount of $\alpha$-helix decreased gradually under both $N_2$ and CO atmospheres (Fig. 4A), and the $\alpha$-helical content under $N_2$ atmosphere at pH 3.3 became the same as that of the unfolded protein within 20 min (Fig. 4).

The rate constant for the faster absorption change under CO atmosphere was about 15 min (Fig. 3) and the shift in the Soret maximum wavelength by interaction with CO at acidic pH was completed in 20–40 min (data not shown). Meanwhile, the $\alpha$-helical content of the protein decreased for about 75% after 20–40 min incubation with CO at acidic pH compared with that of its native form (Fig. 4A, b). These results indicate that CO does delay the unfolding of the protein, but it could not stop the unfolding; the coincidence in these time scales indicates that CO binds to the protein even when the protein is mostly unfolded. Actually, it is reported that CO can bind to the heme iron when cyt *c* is unfolded with Gdn · HCl (20). The delay in the unfolding of the cyt $b_5$ protein by CO indicated its stabilization effect on the protein structure, probably

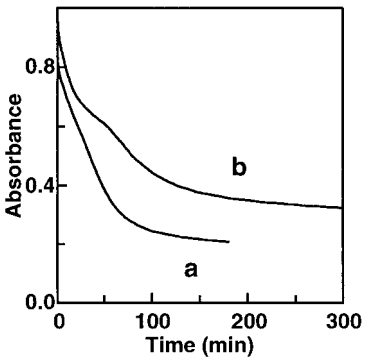


**FIG. 3.** The time courses of the Soret absorption intensity of reduced cyt $b_5$ (7 $\mu$M) at pH 3.46 under $N_2$ (a) and CO (b) atmospheres. Absorption changes were monitored at 423 and 420 nm for measurements under $N_2$ and CO atmospheres, respectively. Measured at 15°C.

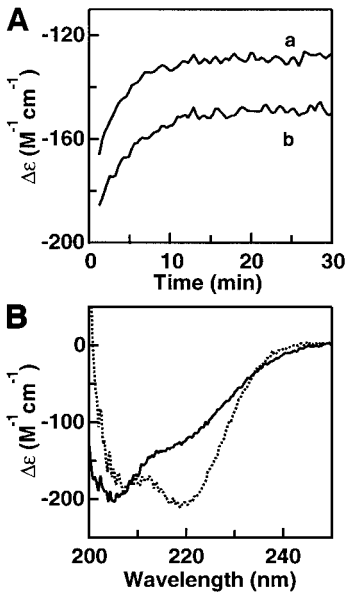


**FIG. 4.** CD ellipticity and spectra of cyt $b_5$. (A) Time courses for the ellipticity at 220 nm under $N_2$ (a) and CO (b) atmospheres at pH 3.3. (B) CD spectra of folded reduced cyt $b_5$ at pH 7.4 (dotted line) and unfolded cyt $b_5$ at pH 3.3 (solid line). Sample concentration was 3 $\mu$M. Measured at 15°C.

around the coordinated heme (hydrophobic pocket), since CO binds stronger to Fe(II) in a hydrophobic pocket than in a hydrophilic environment.

We demonstrate for the first time that CO binds to reduced cyt $b_5$ at acidic pH when the protein starts to unfold, whereas cleavage of the second iron—histidine bond and heme dissociation from the protein are suppressed by CO coordination to the heme iron.

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