# Resonance Raman Studies of $Cu_A$-Modified Cytochrome *c* Oxidase†

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ABSTRACT: Modification of the $Cu_A$ site in mammalian cytochrome *c* oxidase has been used to elucidate the functional role of this center in the catalytic cycle of the enzyme. Both heat treatment in detergents and chemical modification by *p*-(hydroxymercuri)benzoate (pHMB) convert $Cu_A$ to a lower potential type II center and effectively remove the site from the electron-transfer pathway during turnover. In this study, resonance Raman spectroscopy has been employed to investigate the effects of these $Cu_A$ modifications on the heme active sites. The Raman data indicate some environmental perturbation of the heme $a_3$ chromophore in the modified derivatives. Only pHMB modification and SB-12 heat treatment produced significant effects in the Raman spectra of the fully reduced enzyme. These perturbations are much less evident in the spectra obtained within 10 ns of CO photolysis from the fully reduced species of the modified enzymes. Transient Raman studies further indicate that the half-time for CO religation in the modified enzymes is quite similar to that of the native protein.

Cytochrome *c* oxidase, the terminal enzyme in the mitochondrial respiratory chain, mediates the reoxidation of ferrocytochrome *c* by molecular oxygen. Four redox-active metal sites are involved in this important process: two copper centers and two heme A chromophores. The reduction of molecular oxygen takes place at a binuclear cluster containing one of the hemes (designated cytochrome $a_3$) and one of the copper sites (designated $Cu_B$) (Wikstrom et al., 1981; Malmstrom, 1979). The other two metal sites (designated cytochrome *a* and $Cu_A$) have reduction potentials near that of cytochrome *c* during turnover and mediate the electron flow from ferrocytochrome *c* to the binuclear cluster. Since the electrons from ferrocytochrome *c* originate in the cytosol of the mitochondrion and the protons that are consumed in the formation of water from dioxygen are taken from the inner matrix, the redox free energy between ferrocytochrome *c* and molecular oxygen is converted, in part, into a transmembrane protonmotive force during respiration. In addition, concurrent with this electron flow, protons can be vectorially pumped across the inner membrane from the matrix to the cytosol to augment the transmembrane electrochemical gradient produced by the scalar reaction. Thus, cytochrome *c* oxidase is a versatile free energy transducer capable of varying the efficiency of free energy conversion to meet varying power requirements of the mitochondrion (Wikstrom & Saari, 1977; Sigel & Carfoli, 1978).

There is now some evidence that the low-potential metal sites play a role in the proton-translocating function of the enzyme. Both heme *a* and the $Cu_A$ site have been proposed as the site of the redox-linked proton translocation (Callahan & Babcock, 1983; Gelles et al., 1987). To understand the possible role of these low-potential metal centers in proton pumping, we are studying several modified forms of the enzyme. A number of modified forms of cytochrome *c* oxidase are known in which the proton pumping behavior of the enzyme is perturbed. Mild heat treatment of the enzyme in varying detergents or chemical modification by *p*-(hydroxymercuri)benzoate (pHMB)[1] abolishes the proton pumping behavior in cytochrome *c* oxidase while maintaining some level of activity toward electron transfer and dioxygen reduction. Electron paramagnetic resonance (EPR) and optical absorption data demonstrate that, in these variant forms of the enzyme, the local ligand environment of $Cu_A$ was altered while the heme sites were found to be only slightly perturbed (Gelles & Chan, 1985; Nilsson et al., 1988; Li et al., 1988).

In the present study, we employ resonance Raman spectroscopy to provide a corroborative structural probe of the effects of $Cu_A$ modification on the heme active sites of the enzyme. The vibrational spectrum of cytochrome *c* oxidase is complicated by the presence of two heme *a* chromophores. From studies of model compounds and mixed valence inhibitor complexes, it has been demonstrated that spectral isolation of the individual hemes in the enzyme may be obtained by proper selection of excitation wavelengths. In this manner, the equilibrium vibrational structure of the hemes in cytochrome *c* oxidase has been obtained (Babcock et al., 1979, 1981; Babcock, 1988; Choi et al., 1983; Tsubaki et al., 1980; Woodruff et al., 1981; Callahan & Babcock, 1981; Argade et al., 1986). The resonance Raman data presented here clearly indicate that $Cu_A$ modification by heat treatment or pHMB results in only slight perturbation of the heme sites, principally the heme $a_3$ active site in the enzyme.

## Materials and Methods

Bovine cytochrome *c* oxidase was isolated by the method of Hartzell and Beinert (1974) as modified by Li et al. (1988). Samples of $Cu_A$-modified enzyme were prepared as follows: (1) Chemical modification by pHMB was accomplished by adding ~18 mg of pHMB (Sigma) to 10 mL of ~30 μM (per

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[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; pHMB, *p*-(hydroxymercuri)benzoate; SB-12, sulfobetaine-12.

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heme $a_3$) enzyme solubilized in 0.05 M Hepes (pH 7.4)/0.5% lauryl β-D-maltoside containing 50 mM NaCl. After incubation for 1 h at room temperature, the sample was placed on a Sephadex G-25 column and eluted with the Hepes/maltoside buffer system described above to remove unreacted pHMB. (2) The sulfobetaine-12 (SB-12) heat-treated enzyme was prepared by incubating a solution of ~50 μM (per heme $a_3$) enzyme solubilized with 15 mM SB-12 in 0.5 M Hepes (pH 7.4) containing 0.15 M NaCl for 75 min at 43 °C. The solution was then filtered through a 0.45-μm membrane to remove any aggregated enzyme. (3) Heat treatment of the enzyme in lauryl maltoside detergent was carried out in the same manner as in SB-12, except that the detergent system was 0.5% lauryl β-D-maltoside rather than 15 mM SB-12. After heat treatment, the solution was also filtered to remove aggregated enzyme. All samples were concentrated to 100 μM using a MiniCon concentrator (Amicon) with a 20000 molecular weight cutoff. The samples were then frozen and stored at 77 K until needed.

Samples of the fully reduced enzyme for resonance Raman spectroscopy were prepared by degassing 200 μL of the enzyme solution (~100 μM per $a_3$) with five cycles of vacuum/$N_2$ in an anaerobic optical cell. Reduction was accomplished by addition of several grains of solid sodium dithionite (Aldrich) under a $N_2$ atmosphere. To prepare the CO-inhibited enzyme, fully reduced samples were flushed with five cycles of vacuum/CO with a final CO atmosphere of 60 mmHg. Complete reduction and CO ligation were monitored spectrophotometrically at 442 and 430 nm, respectively, on a Hewlett-Packard HP8452A diode array UV/vis spectrometer.

Resonance Raman spectra were obtained with instrumentation described in detail elsewhere (Findsen, 1986). This instrumentation consists of a nitrogen pumped dye laser (Molectron UV-24/DL14), a SPEX 1403 double monochromator, and a SPEX DM3000R controller. Spectra of the resting enzyme were obtained by using 410-nm excitation with slightly defocused cylindrical optics to avoid photoreduction of the enzyme (10-ns pulse width, average power ~3 mW). Spectra of equilibrium fully reduced enzymes were obtained with 440-nm excitation (10-ns pulse width, average power ~6 mW). The 10-ns transient species were generated by exciting a sample of fully reduced CO-bound species with 10-ns pulses (440 nm, 15 Hz, average power ~6 mW). Focused spherical optics were employed for these experiments. Time-resolved Raman spectra were obtained by using a pump/probe protocol. The probe laser (Molectron UV-24/DL14) was triggered at a specific time interval after the pump laser (frequency doubled, Quanta-Ray DCR-11/DL-11) with a digital delay generator (Cal Avionics, 113br).

## Results

The resonance Raman spectra of the pHMB-modified, SB-12 heat-treated, and lauryl maltoside heat-treated enzyme display similarities in the high-frequency region for both the resting oxidized (Figure 1) and fully reduced forms (Figure 2). The decreased signal-to-noise ratio apparent in the spectra of the modified enzymes, relative to the native form, is due to an increase in the fluorescence background from the modified enzymes. The fluorescence is particularly pronounced for the pHMB-modified fully reduced species and, in the case of the pHMB- and SB-12-modified derivatives, also in their resting forms. We observe little or no change in the position and intensity of $\nu_4$ upon modification of the enzyme (1372 $cm^{-1}$ resting, 1355 $cm^{-1}$ fully reduced). However, some small intensity changes are observed in vibrational modes sensitive to the heme A core size (C–N distance). By analogy to heme

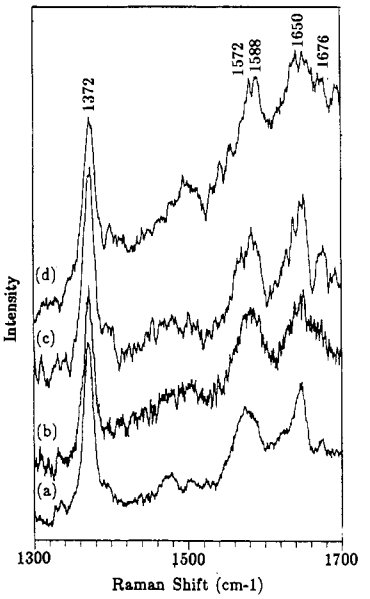


FIGURE 1: Resonance Raman spectra of the resting enzyme in the high-frequency region: (a) native; (b) pHMB-modified; (c) lauryl maltoside heat-treated; (d) SB-12 heat-treated. Spectra are sums of 5–7 scans recorded at 10 $cm^{-1}$/min. Excitation wavelength was 410 nm (average power ≈ 3 mW).

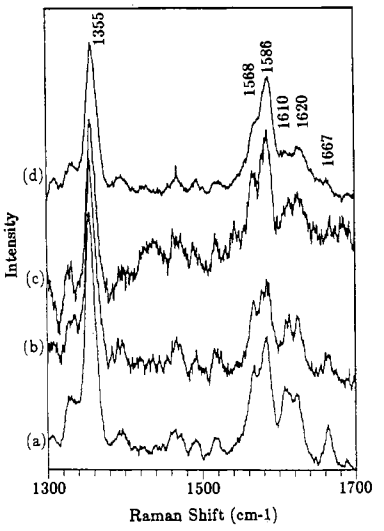


FIGURE 2: Resonance Raman spectra of the fully reduced enzyme in the high-frequency region: (a) native; (b) lauryl maltoside heat-treated; (c) pHMB-modified; (d) SB-12 heat-treated. Experimental conditions were the same as in Figure 1, with the exception of the excitation wavelength which was 440 nm (average power ≈6 mW).

A model compounds, the bands at ~1575 and ~1590 $cm^{-1}$, for both the resting and fully reduced forms of the enzyme, have been assigned to $\nu_2$ of heme $a_3$ (high spin) and $\nu_2$ of heme $a$ (low spin), respectively (Babcock, 1988; Choi et al., 1983). We observe a small shift in intensity from ~1572 to ~1588 $cm^{-1}$ in the resting forms of the various modified derivatives relative to the resting native enzyme. Similarly, the fully reduced modified species also display some enhancement in the intensity of the low-spin $\nu_2$ band, although the magnitude of the increase varies from preparation to preparation. We do not attribute these intensity variations to resonance effects since Cu modification produces only minimal changes in the position, line width, and intensity of the Soret region of the heme absorption.

The fully reduced, modified derivatives, however, exhibit marked intensity changes in the band at 1667 $cm^{-1}$. This band

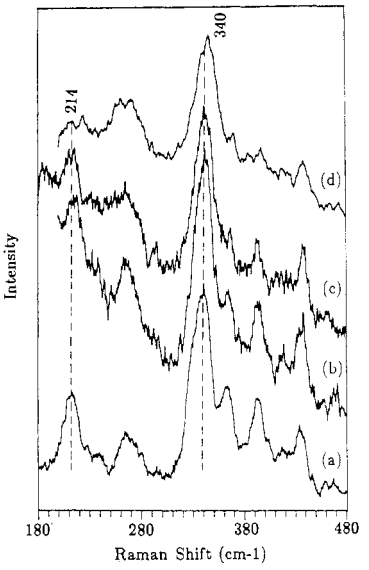


FIGURE 3: Resonance Raman spectra of the fully reduced enzyme in the low-frequency region: (a) native; (b) lauryl maltoside heat-treated; (c) pHMB-modified; (d) SB-12 heat-treated. Experimental conditions were the same as in Figure 2.

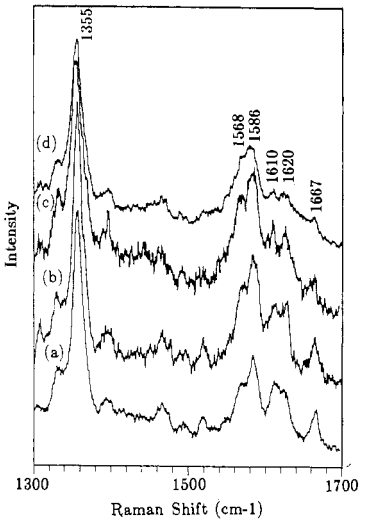


FIGURE 4: Resonance Raman spectra of the 10-ns CO-photolyzed species in the high-frequency region: (a) native; (b) lauryl maltoside heat-treated; (c) pHMB-modified; (d) SB-12 heat-treated. Experimental conditions were the same as in Figure 2.

has previously been assigned to the formyl stretch of heme $a_3$ in a hydrophobic environment (Tsubaki et al., 1980). The intensity of this mode decreases significantly in the pHMB-modified and SB-12 heat-treated enzymes. In the lauryl maltoside heat-treated protein, it is relatively unchanged compared to the native enzyme.

The resonance Raman spectra of the fully reduced enzyme in the low-frequency region obtained with 440-nm excitation are dominated by heme $a_3$ vibrational modes (Figure 3). One mode of particular interest in heme-containing proteins is the Fe–His stretch (214 $cm^{-1}$ for cytochrome *c* oxidase), which is observed only for the five-coordinate, high-spin species (Ogura et al., 1983). The intensity of this mode is lower in all three forms of the modified enzyme. Sone et al. (1986) have also reported a similar diminution of intensity for the Fe–His stretch in the bovine enzyme heat treated in phosphate/cholate buffer.

Figures 4 and 5 show resonance Raman spectra of the native and modified oxidase in the high-frequency (Figure 4) and low-frequency (Figure 5) regions subsequent to ligand photolysis from the CO-bound fully reduced derivatives. Previous studies have established that CO photolysis instantaneously creates a high-spin heme $a_3$ site (on a subnanosecond time scale) with a transient proximal pocket geometry that relaxes to its equilibrium configuration on a microsecond time scale (Findsen et al., 1987). The data depicted in Figures 4 and 5 indicate that the modified enzymes exhibit similar behavior. In all cases, CO photolysis produces a high-spin heme $a_3$ site, as evidenced by the fact that the intensities of $\nu_{Fe-His}$ and the high-spin marker band $\nu_2$ at 1570 $cm^{-1}$ in the transient spectra of the modified samples remain similar to those of the native enzyme. Moreover, the position of $\nu_{Fe-His}$ ($\sim$222 $cm^{-1}$) is the same for all preparations. There are, however, some subtle variations in the spectra above 1600 $cm^{-1}$ among the various preparations. While the photolytic transient of lauryl maltoside heat-treated enzyme yields a spectrum that is very similar to that of the transient species of native oxidase in this region, the corresponding spectra of the photolytic transients of the pHMB- and SB-12-treated samples more closely resemble the equilibrium spectra of the modified species.

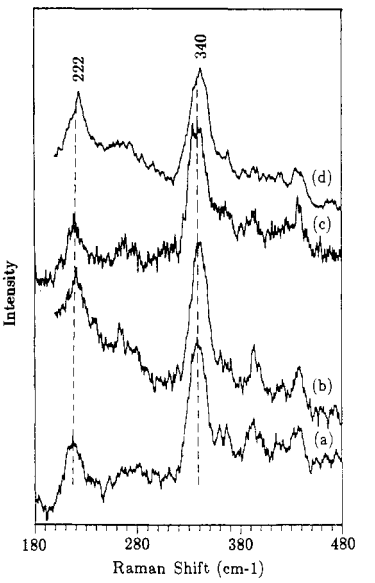


FIGURE 5: Resonance Raman spectra of the 10-ns CO-photolyzed species in the low-frequency region: (a) native; (b) lauryl maltoside heat-treated; (c) pHMB-modified (d) SB-12 heat-treated. Experimental conditions were the same as in Figure 2.

The rebinding of CO subsequent to photolysis can be followed by monitoring the evolution of $\nu_4$. Figure 6 shows that the half-life for CO recombination (indicated by the growth of the ligated $\nu_4$ at 1368 $cm^{-1}$) is greater than 1 ms for the lauryl maltoside heat-treated enzyme. This is consistent with the rebinding kinetics previously reported for the native protein (Findsen et al., 1987). Similar results are obtained for other modified preparations.

## Discussion

The results of the present resonance Raman study of three forms of $Cu_A$-modified oxidase derivatives indicate that the various methods of modification produce some effects at the oxygen binding site (heme $a_3$) whereas the heme *a* chromophore remains relatively unaffected by modification. These effects are most apparent in the SB-12 heat-treated and pHMB-modified forms, which display similar spectral changes relative to the native enzyme. On the other hand, the resonance Raman spectrum of the fully reduced lauryl maltoside heat-treated enzyme is virtually identical with that of the native enzyme. Previous studies have demonstrated that both pHMB

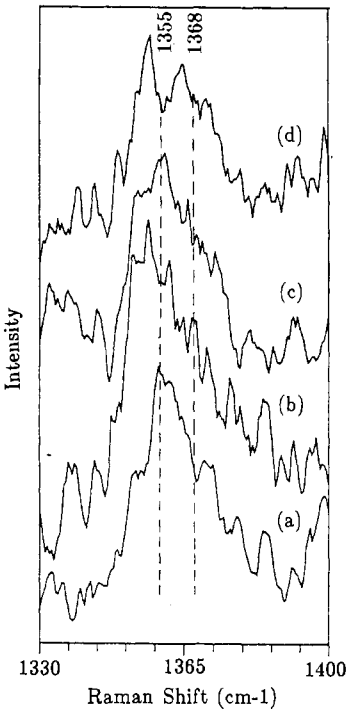


FIGURE 6: Time-resolved resonance Raman spectra of $\nu_4$ subsequent to CO photolysis in the lauryl maltoside heat-treated enzyme: (a) 400 ns; (b) 400 μs; (c) 1 ms; (d) 5 ms. Spectra were obtained by using a low-power probe (440 nm) and a 570-nm pump (see text for details).

modification and mild heat treatment in the zwitterionic detergent SB-12 convert the $Cu_A$ site to a type II copper while heat treatment in lauryl maltoside (nonpolar) gives a distribution of oxidase species consisting of unmodified $Cu_A$, a type II $Cu_A$, and a type I "blue" $Cu_A$ (Nilsson et al., 1988; Li et al., 1988). A connection between the heme $a_3$ heme pocket geometry and $Cu_A$ modification has been suggested by the observation that both the fully reduced and $CN^-$ mixed-valence species are not susceptible to $Cu_A$ modification by heat treatment (Li et al., 1988). Cyanide binding studies have also revealed that reduction of $Cu_A$ and/or heme *a* triggers a conformational transition that affects the heme $a_3$ site, suggesting some allosteric interaction between the low-potential centers and heme $a_3$ (Scholes & Malmström, 1986).

*Equilibrium Structures of Heme Sites in the Modified Enzymes.* The resonance Raman data presented here for the $Cu_A$-modified enzymes indicate that the intensity of the formyl stretching vibration of heme $a_3$ is greatly reduced in the spectra of the fully reduced modified enzymes. Previous work has demonstrated that the band at 1667 $cm^{-1}$ in the fully reduced enzyme can be attributed to a formyl vibration in a hydrophobic environment. This band exhibits a 23 $cm^{-1}$ downshift (to 1644 $cm^{-1}$) upon $CN^-$ binding, indicating that this mode is sensitive to ligation at heme $a_3$ (Babcock, 1988; Choi et al., 1983; Ching et al., 1985). The high-frequency spectra of the fully reduced modified enzymes show no parallel increase in the intensity of the 1644 $cm^{-1}$ band. In addition, there is some evidence suggesting that a fraction of the heme $a_3$ sites are converted to low-spin species in the modified enzymes. This is reflected in the behavior of $\nu_2$ in the spectra. The shift in intensity, from ~1570 to ~1590 $cm^{-1}$, is diagnostic for a high-spin to low-spin transition (Babcock et al., 1979, 1981; Woodruff et al., 1981; Callahan & Babcock, 1981; Argade et al., 1986) and is evident, to varying degrees, in all spectra of the modified enzymes. The data on the fully reduced preparations are more difficult to quantify because of the contributions of $\nu_{37}$ to the observed intensity at ~1570 $cm^{-1}$. Nonetheless, it is clear that both pHMB-treated and SB-12 heat-treated species exhibit an increase in the low-spin band (~1590 $cm^{-1}$) relative to both $\nu_4$ and the combined intensities of $\nu_{37}$ and $\nu_2$ (high spin) at ~1570 $cm^{-1}$. The diminished intensity of the heme $a_3$ Fe–His stretching mode in these preparations corroborates a partial conversion of heme $a_3$ to a low-spin six-coordinate site. Interestingly, these effects are largely absent in the lauryl maltoside heat-treated preparation.

Our data indicate that enzyme modifications produce far less pervasive changes in the local environment of heme *a*. The positions of modes assigned to heme *a* at ~1305 $cm^{-1}$ ($\nu_{21}$), ~1490 $cm^{-1}$ ($\nu_3$), ~1520 $cm^{-1}$ ($\nu_{11}$), ~1590 $cm^{-1}$ ($\nu_2$), ~266 $cm^{-1}$, and ~437 $cm^{-1}$ in the fully reduced enzyme are not significantly affected by any of the modifications. There is some evidence that the formyl stretching mode of heme *a* may be somewhat perturbed. The decreased intensity of the 1610 $cm^{-1}$ band in the fully reduced form of the modified enzymes may be due, in part, to alteration of the heme *a* peripheral environment. However, this band arises from several contributions. Model compound data suggest that $\nu_{10}$ (spin-state sensitive) for reduced, five-coordinate, high-spin heme $a_3$ is located at 1609 $cm^{-1}$ (Babcock, 1988; Choi et al., 1983). Ching et al. (1985) have also reported a decrease in intensity of this mode upon $CN^-$ binding to the fully reduced native enzyme, indicating that this mode is sensitive to the ligation state at the heme $a_3$ site. The 1610 $cm^{-1}$ band has also been shown to be sensitive to H/D substitution, and its position is consistent with the formyl stretch of heme *a* model compounds in polar hydrogen-bonding solvents. Thus, the intensity decrease in this region may originate from contributions from both $\nu_{10}$ for heme $a_3$ and $\nu_{C=O}$ for heme *a*. A similar situation occurs in the 1600–1630 $cm^{-1}$ region of the resting enzyme, where the poorer signal-to-noise ratios of the spectra modified preparations and possibly multiple contributions to the bands from both hemes preclude definitive assignment of the changes to the two heme sites.

The origins of the collective structural perturbations at the heme $a_3$ site are difficult to isolate. The complex changes induced by modification in the 1600–1700 $cm^{-1}$ bands are probably not directly coupled to changes in the heme $a_3$ distal pocket. We conclude that they arise from a more generalized perturbation of the heme $a_3$ peripheral environment. In this respect, the spectra of the fully reduced pHMB-modified and SB-12 heat-treated samples resemble those of alkaline-denatured oxidase (Salmeen et al., 1978). However the lack of significant shifts in either $\nu_4$ (reduced and resting) or $\nu_{Fe-His}$ (reduced) argues against significant perturbation of the proximal pocket for the heme $a_3$ sites of the pHMB-modified and SB-12 heat-treated samples. We speculate that the modifications which induce a conversion of $Cu_A$ to type II copper also perturb the distal pocket of heme $a_3$.

*Photolytic Transient Species.* Transient resonance Raman spectroscopy has recently been used to study ligand binding in hemoglobins and cytochrome *c* oxidase (Babcock et al., 1984; Ogura et al., 1985; Findsen et al., 1987). Data obtained from these studies have yielded valuable information concerning the structural dynamics of the proximal heme pocket and the kinetics of ligand rebinding. The transient spectra of the modified enzymes should, therefore, help in assessing the ramifications of pHMB modification and heat treatment upon structure and reactivity of the cytochrome $a_3$ ligand binding pocket.

Figures 4 and 5 clearly demonstrate that heme $a_3$ assumes a five-coordinate high-spin configuration within 10 ns of CO photolysis from the fully reduced enzyme, irrespective of the

nature of the $Cu_A$ modification. This is evident in the relative intensities of both $\nu_{Fe-His}$ and $\nu_2$ (high spin), which approximate those of the unmodified enzyme.

The position of $\nu_{Fe-His}$ in the 10-ns photolytic transients of the modified enzymes is the same as that of the native protein, indicating a similar metastable geometry of the heme $a_3$ proximal pocket immediately subsequent to CO photolysis. For hemoglobins, the initial position of $\nu_{Fe-His}$ following photolysis has been used to infer the properties of the proximal heme pocket in the ligated species (since little or no relaxation of the protein tertiary structure is assumed to occur on a <10-ns time scale). A similar rationale, when extended to oxidase, argues that little or no perturbation of the proximal heme $a_3$ environment occurs as a result of $Cu_A$ modification.

Interestingly, the photolytic transients of the pHMB-modified and SB-12 heat-treated enzymes both exhibit perturbations of 1600–1700 $cm^{-1}$ bands similar to those observed for their equilibrium fully reduced counterparts. This result further demonstrates that these effects are not linked to the distal pocket of heme $a_3$.

The rate of CO rebinding after photolysis can be monitored by measuring the growth of the ligated $\nu_4$ mode at ~1368 $cm^{-1}$. Previous studies of native, fully reduced oxidase revealed that CO rebinding is quite slow (~5 ms), relative to other heme proteins, presumably because the heme $a_3$ distal pocket is largely open to the solvent (Findsen et al., 1987). Somewhat surprisingly, this is also the case for the pHMB-modified and lauryl maltoside heat-treated enzymes, suggesting that structural perturbations induced at heme $a_3$ by these treatments have a minimal effect upon ligand binding kinetics in the fully reduced enzyme. This contrasts with the inhibition of $CN^-$ binding kinetics observed in the modified resting enzyme (Nilsson et al., 1988).

## Conclusions

In summary, the resonance Raman data provide clear evidence that $Cu_A$ modification causes alterations at the heme $a_3$ site in both the resting and fully reduced enzymes. The data indicate that the perturbations are most pronounced in the modified enzymes in which $Cu_A$ has been converted exclusively to a type II Cu site. Transient resonance Raman data show that the proximal pocket geometry and CO rebinding kinetics of the ferrous heme $a_3$ site remain largely unaltered in the modified forms of the enzyme. This suggests that the physiological integrity of these forms of the enzyme is largely maintained.

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