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Optical and Resonance Raman Spectroscopy of the Heme Groups of the Quinol-Oxidizing Cytochrome *aa*$_3$ of *Bacillus subtilis*[†]

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ABSTRACT: The cytochrome *aa*$_3$-type terminal quinol oxidase of *Bacillus subtilis* catalyzes the four-electron reduction of dioxygen to water. It resembles the *aa*$_3$-type cytochrome-*c* oxidase in using heme A as its active-site chromophores but lacks the Cu$_A$ center and the cytochrome-*c* oxidizing activity of the mitochondrial enzyme. We have used optical and resonance Raman spectroscopies to study the *B. subtilis* oxidase in detail. The α-band absorption maximum of the reduced minus oxidized enzyme is shifted by 5–7 nm to the blue relative to most other *aa*$_3$-type oxidases, and accordingly, we designate the *Bacillus* enzyme as cytochrome *aa*$_3$-600. The shifted optical spectrum cannot be ascribed to an alteration in the strength of the hydrogen bond between the formyl group of the low-spin heme and its environment, as the Raman line assigned to this mode in *aa*$_3$-600 has the same frequency and degree of resonance enhancement as the low-spin heme *a* formyl mode in most other *aa*$_3$-type oxidases. Raman modes arise at 194 and 214 cm^{-1} in *aa*$_3$-600, whereas a single band at about 214 cm^{-1} is assigned to the iron–histidine stretch for the other *aa*$_3$-type oxidases. Possible explanations for the occurrence of these two modes are discussed. Comparison of formyl and vinyl modes and heme skeletal vibrational modes in different oxidation states of *aa*$_3$-600 and of beef heart cytochrome-*c* oxidase shows a strong similarity, which suggests conservation of essential features of the heme environments in these oxidases.

Bacillus subtilis is a Gram-positive bacterium that forms endospores. It has been shown to have two terminal oxidases of the cytochrome *aa*$_3$-type, the expression of which seems to be controlled by the oxygen tension and the carbon source (Lauraeus et al., 1991). *B. subtilis* grown on a fermentable carbon source and with moderate aeration expresses only one of these *aa*$_3$-type oxidases, here called cytochrome *aa*$_3$-600. This enzyme differs considerably from the *aa*$_3$-type cytochrome-*c* oxidases in mitochondria and some bacteria. In particular, the *aa*$_3$-600 has no cytochrome-*c* oxidase activity, but it does oxidize quinols. In this regard, it is similar to the cytochrome *o* of *Escherichia coli*. The second cytochrome *aa*$_3$-type oxidase in *B. subtilis*, which is not considered here, is a cytochrome-*c* oxidizing enzyme with covalently bound *c*-type heme (*caa*$_3$; Lauraeus et al., 1991) and with an established primary structure that is strongly related to other terminal oxidases of a superfamily of enzymes that includes both the *aa*$_3$-type enzymes from mitochondria and bacteria and the cytochrome *o* from *E. coli* [see Saraste (1990) for a review].

The *aa*$_3$-600 has two A-type hemes, one low-spin and one high-spin, called heme *a* and heme *a*$_3$, respectively, as in conventional cytochrome *aa*$_3$-type enzymes. The reduced enzyme has an exceptional α-band absorption maximum that is 5–7-nm blue-shifted relative to most other oxidases of the *aa*$_3$-type (see Figure 1). The oxidized enzyme does not contain any EPR-visible copper, indicating that the Cu$_A$ center typical of cytochrome-*c* oxidases of the *aa*$_3$-type is missing. This observation is supported by metal analysis of *aa*$_3$-600, which revealed the presence of only one copper per *aa*$_3$ unit. This is probably the Cu$_B$ of the bimetallic heme–copper dioxygen reduction site, in which high-spin heme iron and copper are magnetically coupled (Lauraeus et al., 1991). The lack of the Cu$_A$ site in the cytochrome *aa*$_3$-600 may be related to the enzyme's function as a quinol oxidase rather than as a cytochrome-*c* oxidase. Analogously, the ubiquinol-oxidizing cytochrome *o* of *E. coli* also lacks Cu$_A$ (Puustinen et al., 1991), as does the quinol-oxidizing terminal oxidase from *Sulfolobus acidocaldarius* (Anemuller & Schafer, 1990).

Comparison of *aa*$_3$-600 to other cytochrome *aa*$_3$-type enzymes, and especially to the mitochondrial cytochrome-*c* oxidase, may provide means by which to identify conserved structural features, which can be assumed to be involved in basic functions common to all terminal oxidases. In contrast, dissimilarities between these enzymes are likely to be involved in the fine-tuning of the enzyme to specific needs demanded by differences in local biological environments.

In the work presented here, the heme sites of *aa*$_3$-600 have been characterized by optical and resonance Raman spectroscopies. The results have been compared with the data on the well-studied cytochrome *aa*$_3$ from bovine heart mitochondria [reviewed by Babcock (1988)]. The resonance Raman technique is a powerful method to probe heme structures and yields data on both the oxidized and reduced states of these chromophores that complement insights available from EPR spectroscopy (Lauraeus et al., 1991). We have further compared our results to recent vibrational studies on plant cytochrome-*c* oxidase (de Paula et al., 1990) and *S. acidocaldarius* quinol oxidase (Hildebrandt et al., 1991). Interestingly, the heme structures of *B. subtilis aa*$_3$-600 are in many respects more similar to those of the bovine heart

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enzyme than to those in plant mitochondria or the archaebacterium *Sulfolobus*. This is in good agreement with the recent finding (Santana et al., 1992) that the primary structure of the *B. subtilis* aa_3-600 is closely related to the bovine heart aa_3 and the cytochrome *o* from *E. coli*.

MATERIALS AND METHODS

Bacillus subtilis (strain 168) was grown at 38 °C in a 300-L fermentor in medium containing (w/v) 2.0% glucose, 0.5% yeast extract, 0.5% tryptone, 0.8% ammonium sulfate, 2.0% sodium phosphate, and 0.05% sodium citrate. Cells were harvested in the stationary state. Membranes and aa_3-600 were prepared as described in Lauraeus et al. (1991), except a HiLoad-Q Sepharose column (Pharmacia LKB) was used in the final purification step instead of the mono-Q HR 10 column.

For optical spectroscopy, a stock solution of cytochrome aa_3-600 (usually about 100 μM) was diluted in buffer containing 100 mM MOPS,[1] pH 7.4, 1 mM EDTA, and 0.2% dodecyl saccharide. Oxidized cyanoenzyme ($a^{3+}a_3^{3+}$-CN) was prepared by incubating the oxidase in 1 mM KCN overnight. The mixed valence cyanoenzyme ($a^{2+}a_3^{3+}$-CN) was prepared by adding 15 mM ascorbate and 1 mM TMPD to the oxidized cyanoenzyme. A few grains of solid sodium dithionite, 15 mM ascorbate, and 1 mM TMPD were added to the oxidized enzyme ($a^{3+}a_3^{3+}$) to prepare the fully reduced form ($a^{2+}a_3^{2+}$). The relatively high concentration of TMPD required for reduction of the enzyme is necessitated by the fact that cytochrome aa_3-600 is a quinol oxidase and lacks $\mathrm{Cu_A}$; its reduction, by traditional cytochrome oxidase reductants, is slow. The fully reduced cyanide-bound enzyme ($a^{2+}a_3^{2+}$-CN) was prepared by adding 50 mM KCN to the fully reduced form.

Resonance Raman spectra were obtained from an 80–100 μM sample in 50 mM Tris buffer, pH 7.4, in a cylindrical quartz spinning cell or in 2-mm cuvettes maintained at 2–5 °C by a stream of cold nitrogen gas. The Raman spectra were acquired by using a SPEX 1877 triplemate with an EG&G Model 1421 diode array detector or with a SPEX 1401 spectrometer with a cooled PMT detector (RCA 31034A). A Coherent Innova 90 Krypton ion laser and a Liconix 4240 helium–cadmium laser were used to provide excitation wavelengths of 413.1 and 441.6 nm, respectively. The power incident on the oxidase samples was typically 10–35 mW.

RESULTS

Optical Absorption Spectra. The reduced minus oxidized absorption difference spectrum of cytochrome aa_3-600 in the visible region is shown in Figure 1. The overall shape of the difference spectrum is similar to that of the mitochondrial enzyme; in contrast to this latter species, however, the difference absorption maximum of the *Bacillus* enzyme is blue-shifted and occurs at 600 nm. Figure 2 provides a comparison of the visible and Soret spectra of the forms of the enzyme studied here [oxidized (Figure 2a), reduced (Figure 2d), and the cyanide-bound enzyme in its fully oxidized (Figure 2b), mixed valence (Figure 2c), and fully reduced (Figure 2e) states]. Most of the spectral changes upon reduction and ligand binding are very similar to those reported previously for beef heart cytochrome-*c* oxidase by Van Buuren et al. (1972).

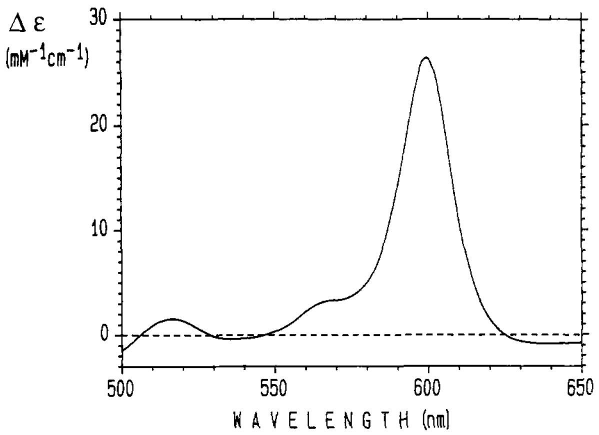

FIGURE 1: Reduced minus oxidized absorption spectrum of aa_3-600. Excess ferricyanide was added to the sample before recording the oxidized spectrum and was followed by the addition of a few dithionite grains to reduce the sample. Heme concentration was measured as described by Berry and Trumpower (1987) and was used to convert the absorption difference to the absorptivity difference scale.

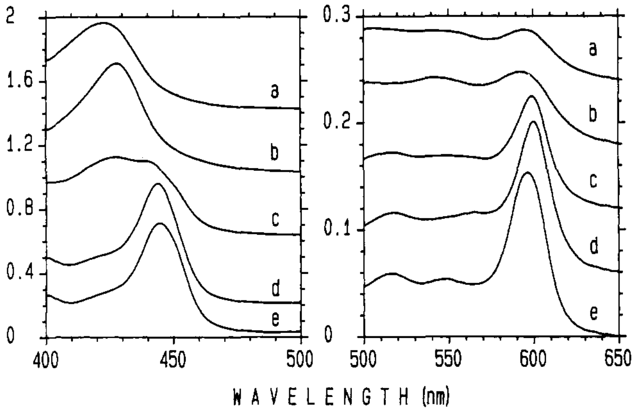

FIGURE 2: Optical absorption spectra of various forms of aa_3-600: (a) air-oxidized aa_3-600; (b) cyanide-ligated form of air-oxidized aa_3-600; (c) mixed valence, cyanide-ligated form of aa_3-600; (d) dithionite-reduced aa_3-600; (e) dithionite-reduced, cyanide-ligated form of aa_3-600. The aa_3-600 concentration was 5.3 μM in each sample. For ease of presentation, the absorption spectra have been arbitrarily displaced along the absorbance axis.

The millimolar absorptivity of the reduced minus oxidized enzyme difference spectrum at 600 minus 630 nm is close to 13.5 $\mathrm{cm^{-1}}$ based on total heme (27 $\mathrm{mM^{-1}\ cm^{-1}}$ on an aa_3 basis). This is very similar to the corresponding absorptivity of the bovine enzyme. The ratio of the Soret/α-band absorbance is 5.6, which is also similar to that of the bovine enzyme. These observations, together with the spectra in Figure 2, indicate that the low-spin heme *a* is the dominant absorber in the α-band region in aa_3-600, as it is in the mitochondrial enzyme, and that perturbations to this chromophore are primarily responsible for the absorption band shift.

In earlier studies, it was shown that the absorption maximum of the α-band of reduced heme A model compounds correlates with the strength of the hydrogen bond between hydrogen donors in solution and the formyl group of heme A (Babcock & Callahan, 1983). On the basis of resonance Raman studies, it was further shown in the bovine enzyme that the low-spin heme *a* probably forms a hydrogen bond to an amino acid residue; moreover, this bond appears to be stronger in reduced heme *a* than in the oxidized form. It was proposed that this hydrogen bonding may be responsible for the large, red shift of the α-band in cytochrome aa_3-type enzymes, relative to heme A of model compounds (Babcock & Callahan, 1983).

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[1] Abbreviations: MOPS, 4-morpholinepropanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

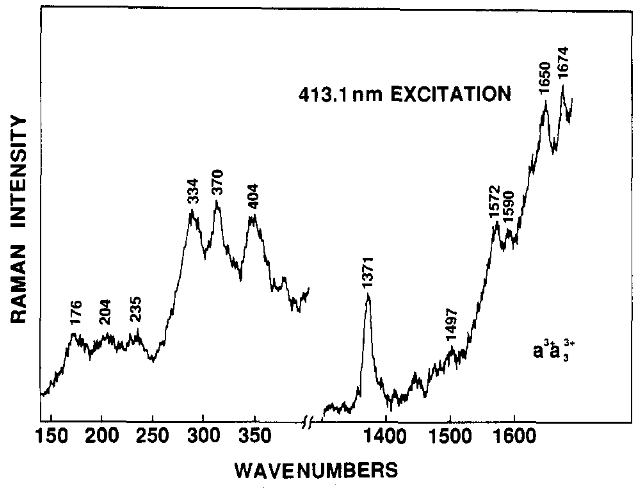

FIGURE 3: Resonance Raman spectra of oxidized aa_3-600. The exciting laser wavelength was 413.1 nm, and the sample was flushed with oxygen to prevent photoreduction of heme *a*.

Table I: Assignment of High-Frequency Vibrations in *Bacillus* aa_3-600

mode	a^{3+}	a_3^{3+}	a_3^{3+}·CN	a^{2+}	a_3^{2+}	a_3^{2+}·CN
ν (C=O)	1650	1674	1671	1611	1666	1644
ν (C=C)			1620	1620	1620	1620
ν_2	1590	1572	1584	1585		1585
ν_{38}			1544	1548	1548	1544
ν_{11}			1516	1517		1517
ν_3			~1503	1492	1468	1493
ν_4	1371	1371	1369	1358	1358	1359
$\nu_{29/20}$				1395	1414	1416
$\delta_s = CH_2$			1331	1290		
ν_{21}				1307		

Below, we will explore whether the blue-shifted α-band of aa_3-600 might be due to alteration of this hydrogen bonding.

Resonance Raman Spectroscopy. Using two different excitation frequencies, it is possible to selectively enhance heme *a* and heme a_3 modes in different redox and ligand states of aa_3-600 (Babcock et al., 1981; Woodruff et al., 1981; Callahan & Babcock, 1983; Ching et al., 1985). The 413.1-nm excitation frequency can be used to enhance the a^{3+}, a_3^{3+}, and a_3^{3+}-CN modes, whereas the 441.6-nm laser frequency enhances a^{2+}, a_3^{2+}, and a_3^{2+}-CN modes. In this way, one can preferentially study one heme at a time and assign its vibrational modes. The analogy we have found between the resonance Raman spectra of the well-characterized mammalian cytochrome-*c* oxidase (Babcock, 1988) and the aa_3-600 has helped the mode assignments given below.

Figure 3 shows the Raman spectrum of fully oxidized aa_3-600 obtained by using 413.1-nm excitation frequency, in which resonances of both hemes are enhanced. A fluorescence background is apparent in the high-frequency region, which is, however, substantially less severe than encountered with the aa_3 quinol oxidase from *Sulfolobus* (Hildebrandt et al., 1991). The 1371-cm^{-1} ν_4 mode from both hemes is pronounced in Figure 2, and ν_2 modes at 1572 (heme a_3) and 1590 (heme *a*) are apparent. The carbonyl vibrations of the two hemes at 1650 (*a*) and 1674 (a_3) cm^{-1} are also clearly resolved. The frequencies of these modes are summarized in Table I and are identical, within experimental uncertainty, to those that occur in the beef enzyme. The most intense modes seen in the low-frequency region are at 334, 370, and 404 cm^{-1}. Like mammalian cytochrome-*c* oxidase, these aa_3-600 modes are shifted 5–8 cm^{-1} from the corresponding modes in heme A model compounds (Callahan et al., 1981; Choi et al., 1983; Babcock, 1988), due to interaction of the peripheral substituents of heme A with the protein (Abe et al., 1978; Choi & Spiro, 1983). The overall similarity between the frequencies and relative enhancements for the vibrational resonances of cytochrome-*c* oxidase and aa_3-600 indicates that the protein milieu surrounding the hemes is similar in these two enzymes.

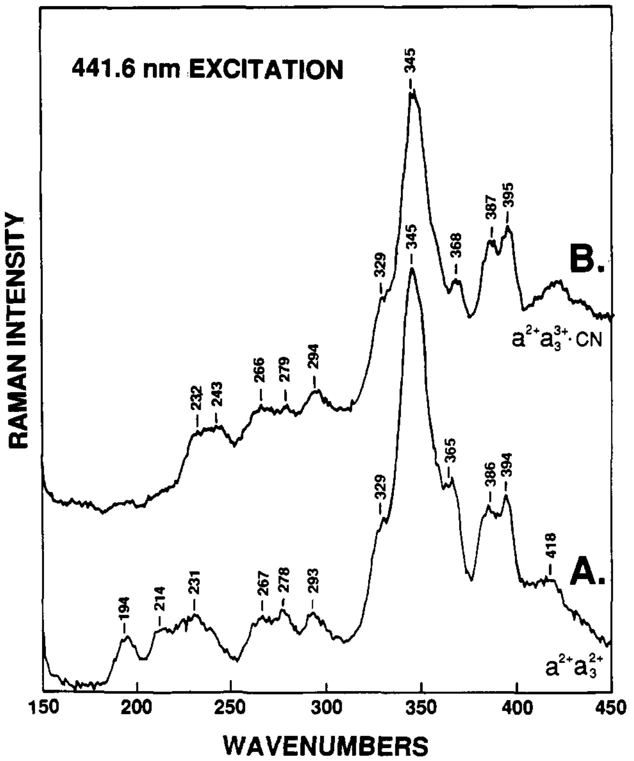

FIGURE 4: Low-frequency resonance Raman spectra of (A) dithionite-reduced aa_3-600; (B) mixed valence cyanide-bound complex of aa_3-600. The exciting laser wavelength was 441.6 nm.

Reduction of iron causes changes in the porphyrin macrocycle geometry, as well as in the bond lengths and angles of substituents conjugated to the heme. Accordingly, reduction shifts the Raman bands of the oxidized heme to new positions; moreover, some new modes are enhanced after reduction. Addition of cyanide to oxidized or reduced cytochrome oxidase causes a high-spin to low-spin transition in heme a_3; the valence and spin state of heme *a* are not altered. Therefore, frequency shifts and intensity changes in Raman bands after cyanide addition can be associated with modes that are sensitive to the spin and valence of heme a_3 (Salmeen et al., 1978; Ching et al., 1985).

Figures 4 and 5 show the resonance Raman spectra of reduced aa_3-600 and the mixed valence cyanide complex ($a^{2+}a_3^{3+}$-CN) in the low- and high-frequency regions, respectively. Frequencies and mode assignments are summarized in Table I. The spectra in both figures were obtained with 441.6-nm excitation, which is in resonance with the Soret transition of reduced heme *a* and of reduced unliganded heme a_3. Therefore, in the reduced enzyme (Figures 4A and 5A), both heme chromophores contribute to the Raman spectrum, whereas only heme *a* modes only are strongly enhanced in the mixed valence cyanide complex (Figures 4B and 5B). An important Raman band in the low-frequency region of the reduced bovine enzyme is found at 214 cm^{-1} and originates from the stretching vibration of the heme a_3 iron – proximal histidine bond (Salmeen et al., 1978; Ogura et al., 1983). This band arises at 214 cm^{-1} in aa_3-600 as shown in Figure 4A; in addition, a new mode of similar intensity has appeared at 194 cm^{-1} in the spectra of the reduced *B. subtilis* enzyme. The

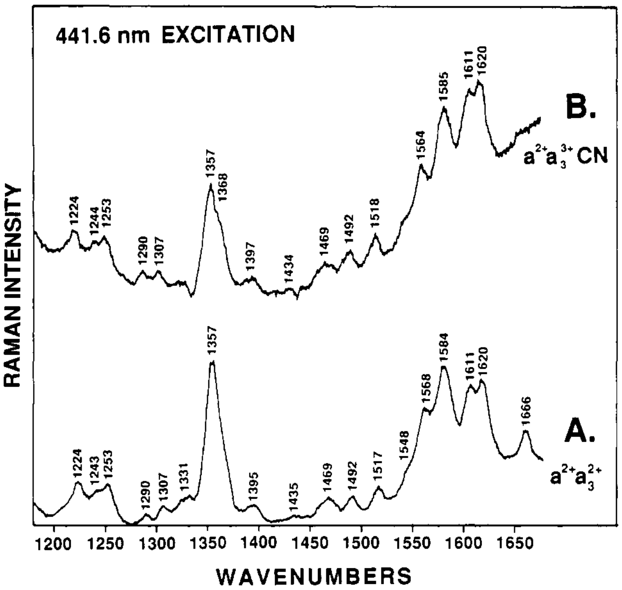

FIGURE 5: High-frequency resonance Raman spectra of (A) dithionite-reduced aa_3-600; (B) mixed valence cyanide-bound complex of aa_3-600. The exciting laser wavelength was 441.6 nm.

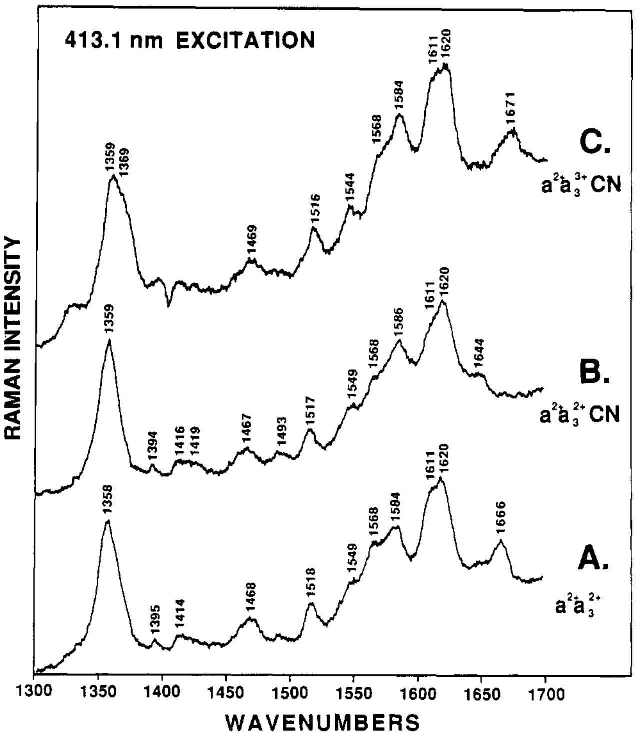

FIGURE 6: High-frequency resonance Raman spectra of (A) dithionite-reduced aa_3-600; (B) dithionite-reduced cyanide-bound complex of aa_3-600; (C) mixed valence cyanide-bound complex of aa_3-600. The exciting laser wavelength was 413.1 nm.

corresponding Raman spectrum of the mixed valence cyanide complex (Figure 4B) shows that the 194, 214, and 365-cm^{-1} bands have disappeared and that no new lines have appeared. Therefore, these lines can be assigned to reduced heme a_3. Their identity is further discussed below.

Other principal bands seen in Figures 4 and 5 represent skeletal modes of reduced and mixed valence aa_3-600 and are assigned as analogous to the vibrations of beef heart cytochrome-*c* oxidase. The most intense feature in Figure 5A is the 1357-cm^{-1} band assigned to the ν_4 skeletal mode. This band arises primarily from the C$_a$–N bond stretching motion of the porphyrin macrocycle and is sensitive to the strength of the porphyrin macrocycle bonds. These, in turn, are functions of the electron density in the porphyrin π^*-orbital. The oxidation state of the heme iron and the identity of its proximal ligands are the most important factors that affect the porphyrin π^*-orbital density and thereby the Raman frequency of ν_4 (Anzenbacher et al., 1989). Comparison of the ν_4 mode in aa_3-600 with beef heart oxidase shows similar frequencies in the fully reduced oxidases; as noted above, identical ν_4 frequencies are also observed in the fully oxidized enzyme spectra. Thus, similar proximal ligation and protein environments of both hemes in both oxidation states may be inferred.

Previous resonance Raman studies of aa_3-type oxidases have shown that resonances originating from heme substituents, mainly formyl and vinyl, are the most variable modes in these oxidases (Babcock, 1988; de Paula et al., 1990; Hildebrandt et al., 1991). In the reduced state (Figure 5A), aa_3-600 exhibits two modes, at 1611 and 1666 cm^{-1}, that arise from formyl stretching vibrations of hemes a and a_3, respectively. In the mixed valence cyanide complex (Figure 5B), the 1666-cm^{-1} band has disappeared, which shows that this line can be attributed to the formyl stretching mode of heme a_3 in the aa_3-600 enzyme. The 1620-cm^{-1} line (Figure 5, panels A and B) can be assigned to the vinyl vibration of both hemes (Callahan & Babcock, 1983; Ching et al., 1985).

Figure 6 shows the Raman spectra of reduced, fully reduced cyanide-bound, and mixed valence cyanide-bound enzyme obtained with 413.1-nm excitation. This laser excitation frequency mainly enhances modes from ferric hemes, but heme a_3^{2+} modes are also observed. The ν_4 mode of aa_3-600 in the mixed valence cyanide-bound complex (Figure 6C) is composed of two bands, one at 1359 cm^{-1} from reduced heme a and one at 1369 cm^{-1} from the oxidized a_3–cyanide complex (Babcock et al., 1981). The formyl stretching mode from low-spin, oxidized heme a_3 is observed at 1671 cm^{-1}, as in beef heart cytochrome-*c* oxidase. In the reduced enzyme, cyanide ligation to high-spin heme a_3^{2+} produces the low-spin $a_3^{2+}\cdot$CN$^-$ complex. This spin-state change shifts the formyl mode from 1666 cm^{-1} in a_3^{2+} to 1644 cm^{-1} in $a_3^{2+}\cdot$CN (Figures 6, panels A and B). Similar shifts of the formyl vibration of heme a_3^{2+} are reported for bovine cytochrome-*c* oxidase (Ching et al., 1985).

DISCUSSION

Optical Spectrum and Formyl Modes. Heme A model compound studies reveal that heme a in aa_3-type enzymes has a red-shifted α-band absorption maximum and that its formyl stretching mode in the Raman spectrum is shifted to lower frequency (Van Steelandt-Frentrup et al., 1981; Callahan & Babcock, 1983). The origin of both of these shifts was suggested to be strong hydrogen bonding between the formyl oxygen and an amino acid hydrogen donor in the protein (Babcock & Callahan, 1983) or, alternatively, resonance energy transfer with water molecules in the vicinity (Sassaroli et al., 1989). Furthermore, it was shown that reduction of heme a strengthened the hydrogen bond, and this redox-dependent interaction was suggested to be involved in the mechanism of proton translocation (Babcock & Callahan, 1983). In recent work, it has been shown that aa_3-type oxidases isolated from plant mitochondria (de Paula et al., 1990) and from *S. acidocaldarius* (Hildebrandt et al., 1991) have blue-shifted reduced minus oxidized α-bands. They were also shown to have apparently weaker hydrogen bonds between the chromophore formyl group and the protein in the reduced enzyme, to which the optical absorption band shift was attributed.

The optical absorption spectrum of aa_3-600 also has a blue-shifted α-band in comparison to mammalian cytochrome-*c* oxidase, and a primary motivation for the present work was to determine whether the optical shift in aa_3-600 can be explained in the same fashion. The results presented above, however, show that the formyl stretching modes of oxidized and reduced heme *a* of aa_3-600 have Raman frequencies identical to those in mammalian cytochrome-*c* oxidase. This indicates that the hemes *a* in the two enzymes have similar environments and interactions in their respective formyl groups. We conclude that the blue shift of the α-band in aa_3-600 cannot be attributed to weakening of the interaction of the formyl group with its environment. Neither EPR (Lauraeus et al., 1991) nor resonance Raman spectroscopy (this work) reveal any changes in the heme *a* environment relative to the bovine enzyme that could explain the spectral shift. This suggests that the origin of the optical perturbations in the *Bacillus* enzyme may be associated primarily with local protein effects on excited-state energies rather than in modifications to the ground-state potential surface. It is interesting that the mammalian and *B. subtilis* oxidases use different electron donors, and the latter lacks the Cu_A center, and yet both oxidases have the same conserved formyl group of heme *a*, the interaction of which with the environment is sensitive to the redox state of the heme. This suggests a functional role for the formyl–protein interaction in these oxidases. However, this specific heme–protein interaction is clearly absent in the quinol-oxidizing, cytochrome *o* class of terminal oxidases, the hemes of which lack the formyl group (Puustinen & Wikström, 1991; Wu et al., 1992).

The formyl group of heme a_3 is not as profoundly sensitive to oxidation state as that of heme *a*, and that of aa_3-600 seems to be similar to the aa_3-type oxidases studied so far (Callahan & Babcock, 1983; Ogura et al., 1984; de Paula et al., 1990; Hildebrandt et al., 1991). The frequencies of the heme a_3 formyl bands of both oxidized and reduced aa_3-600 are identical to the corresponding vibrations in the oxidases from the various sources noted above. Moreover, in the *Bacillus* enzyme, the formyl stretching mode of heme a_3 is sensitive to the spin state of the iron, as it is in the mammalian enzyme (Ching et al., 1985). These similarities indicate a conserved formyl environment of heme a_3 in all aa_3-type oxidases, such that the formyl of this chromophore is not involved in hydrogen bonding (Van Steelandt-Frentrup et al., 1981), and imply that hydrophobicity, at least in the region around the heme periphery, is characteristic of the binuclear center binding pocket. Similar inferences regarding the binuclear center have also been drawn from infrared spectroscopy of the bovine enzyme (Einarsdottir et al., 1988).

Vinyl Modes. The C=C double bond of the vinyl group is not strongly conjugated to the heme macrocycle, and therefore its resonance enhancement in Soret excitation heme Raman spectra is somewhat surprising. Dipole–dipole interaction of the vinyl double bond and the heme macrocycle has been proposed as the mechanism for this enhancement [Tobias, 1981; see, also, Devito and Asher (1989)]. Studies of vinyl-substituted porphyrin model compounds show that variation in the vinyl–porphyrin angle can change the C=C vibration frequency (Bocian et al., 1986; Gersonde et al., 1989). The two reduced hemes of aa_3-600 have identical vinyl vibration frequencies relative to mammalian cytochrome-*c* oxidase, indicating similar orientations of the vinyl group with respect to the hemes in these two oxidases. Conservation of the vinyl–porphyrin angle in the oxidases can be assumed to be due to conserved vinyl interaction domains in the protein, which contribute to the correct orientation of the hemes in the enzyme. In contrast, the vinyl–porphyrin angle is different in plant cytochrome-*c* oxidase, causing a frequency upshift of the vinyl mode that is more apparent for heme *a* (de Paula et al., 1990).

Porphyrin Skeletal Modes. The strong resemblance between resonance Raman spectra of aa_3-600 and mammalian cytochrome-*c* oxidase in their various oxidation and spin states is mostly due to similarities in the frequencies and intensities of their porphyrin skeletal vibrational modes. These are sensitive to the electron density at the heme irons, the core size of the porphyrin, and to interactions of porphyrin substituents with their protein environment (Abe et al., 1978; Callahan & Babcock, 1981; Ozaki et al., 1986; Babcock, 1988). The spectroscopic similarity of the heme chromophores in these two oxidases suggests a strong similarity of ligation and heme environment of the *a* and a_3 centers in aa_3-600 and in the bovine enzyme. Recent sequence analysis work with several related quinol and cytochrome oxidases [see Saraste (199) and Babcock and Wikström (1992) for reviews] and directed mutagenesis on the *E. coli* cytochrome *o* (Lemieux et al., 1992; Minagawa et al., 1992) and the *R. sphaeroides* cytochrome oxidase (Shapleigh et al., 1992) have delineated the low-spin and high-spin heme binding sites in this class of enzyme. The data presented here indicate that the aa_3-600 enzyme has heme sites consistent with its classification as a member of this superfamily. This conclusion was recently substantiated by the elucidation of the operon of the aa_3-600 enzyme (Santana et al., 1992). The structural genes were found to strongly resemble those of the oxidase family, in particular those of cytochrome *o* of *E. coli.*

Iron–Histidine Stretching Mode of Heme a_3. Salmeen et al. (1978) and Babcock et al. (1981) have assigned the 214-cm^{-1} line in reduced beef heart oxidase to a heme iron–histidine stretching vibration of a_3^{2+}. Subsequently, the same resonance was found in both yeast and bacterial aa_3-type cytochrome-*c* oxidases (Ogura et al., 1984). Isotopic iron substitution experiments by Ogura et al. (1983) as well as heme A model compound studies by Van Steelandt-Frentrup et al. (1981) have confirmed this assignment. In fully reduced aa_3-600, the 214-cm^{-1} mode is observed, but an additional vibration, at 194 cm^{-1}, that is not observed in the beef heart enzyme is also apparent (Figure 4). Neither the 214-cm^{-1} mode nor the 194-cm^{-1} mode is present in the oxidized or mixed valence cyanide derivative of aa_3-600, verifying that both arise from ferrous heme a_3^{2+}.

A plausible explanation for the occurrence of these two modes is that they arise from the Fe–His stretching vibration of heme a_3^{2+} in two different conformers of the enzyme. A split iron–histidine stretching mode has been assigned in the Raman spectra of *Thermus thermophilus* ba_3 oxidase (Einarsdottir et al., 1989) and in cytochrome-*c* peroxidase (Smulevich et al., 1988). In both enzymes, the two stretching modes are proposed to arise from two different heme–protein conformations that affect the iron–histidine bond strength.

In the case of cytochrome-*c* peroxidase, crystal structure analysis shows that the proximal histidine (His-175) interacts at its N_δ position with a carboxylate hydrogen acceptor on a nearby aspartate residue (Asp-235) (Finzel et al., 1984). The strength of the interaction was proposed to vary according to a double-well potential that produced either deprotonation or hydrogen bonding at the His-175 N_δ position. This variation in protonation state was invoked to account for the occurrence of the split Fe–His-175 stretching mode, which has components at 233 and 246 cm^{-1} in the native enzyme (Smulevich et al., 1988). Upon mutagenesis of Asp-235 to asparagine, the split Fe–His mode was replaced by a single mode at 205 cm^{-1}, which, on the basis of model compound work (Stein et al., 1980; Hori & Kitagawa, 1980; Van Steelandt-Frentrup et al., 1981), was interpreted to indicate elimination of perturbation at the His-175 N$_\delta$H position, i.e., to reflect a non-hydrogen-bonded structure (Smulevich et al., 1988).

An analogous hydrogen-bonded structure that exists in two different conformational states may occur in the aa_3-600 enzyme. From the model compound work, the conformer with the weaker (or absent) hydrogen bond to the proximal histidine is expected to have the weaker iron–histidine bond and the lower frequency vibration, 194 cm^{-1}. In this interpretation, the more strongly hydrogen-bonded conformer in aa_3-600 contributes the 214-cm^{-1} mode and has an iron–histidine bond of strength comparable to that in mitochondrial cytochrome-*c* oxidase. In testing this model, we found that the relative intensities of the low-frequency modes of aa_3-600 did not change significantly in the pH range 5.5–8 (data not shown). However, the proximal protein pocket in heme a_3 of beef heart oxidase appears to be hydrophobic and inaccessible to solvent as indicated by Raman studies in D_2O (Argade et al. 1986a,b; Schoonover et al., 1988). A similar protein environment is likely for aa_3-600, and consequently, the invariance of the intensities of the low-frequency modes of aa_3-600 does not necessarily rule out a hydrogen bond to the proximal histidine of a_3^{2+}.

The angle of the histidine relative to the heme nitrogens is a second factor that may influence the strength of the iron–histidine bond. In the case of *T. thermophilus* ba_3 oxidase, Einarsdottir et al. (1989) have suggested that the two iron–histidine modes observed at 210 and 193 cm^{-1} are due to rotamers of the imidazole ring plane relative to the heme N–Fe–N axis. They found that the relative intensities of the two modes changed with temperature over a broad range (300–10 K), indicating two states in equilibrium. An analogous situation is thought to occur in hemoglobin, where, in the *T* state, a strained iron–histidine conformation is assumed to weaken the iron–histidine bond and lower its stretching frequency (Rousseau & Friedman, 1988).

A third plausible interpretation of the low-frequency aa_3-600 Raman spectrum can be constructed in which conformational heterogeneity is not necessary. In this model, only one of the two modes is assigned as the Fe–His stretch; the second is assigned as the torsional vibration of the histidine ring relative to the heme plane. In molecular dynamics simulations on myoglobin, Petrich et al. (1991) fround that the Fe–heme distance oscillated with a period of 0.2 ps, which corresponds to 170 cm^{-1}. The oscillation was attributed to a libration of the imidazole ring about an axis perpendicular to the heme plane. A low-frequency mode observed in deoxymyoglobin at 150 cm^{-1} has been assigned to the histidine torsional mode (Wells et al., 1991). Thus, the new band observed in aa_3-600 at 194 cm^{-1} could be the histidine ring torsional vibration, while the iron–histidine stretching vibration can be assigned to 214 cm^{-1}, as expected for aa_3-type oxidases.

At present, we are unable to make a definitive assignment of the low-frequency aa_3-600 spectrum. Nonetheless, the data presented here, as well as the earlier work by Einersdottir et al. (1989) on the ba_3 oxidase, show that significant differences in the Fe–His interaction at the a_3 site occur in the cytochrome and quinol oxidase family of enzymes. Variations in the Fe–His interaction at the heme *a* site have also been documented for the *Paracoccus* (Albracht et al., 1980) and *Sphaeroides* (Hosler et al., 1992) enzymes. These observations indicate that the heme–protein contacts at both chromophores can be modulated by local protein effects.

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Registry No. Cytochrome aa_3 quinol oxidase, 69671-26-7; heme *a*, 57560-10-8; heme a_3, 58916-42-0; cytochrome oxidase, 9001-16-5.