Unusual Heme Structure in Cytochrome aa_3 from *Sulfolobus acidocaldarius*: A Resonance Raman Investigation†

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ABSTRACT: Well-resolved Soret-excited resonance Raman spectra have been obtained in the heme marker band region (1440–1700 cm^{-1}) for the fully oxidized and fully reduced forms of the terminal oxidase aa_3 from *Sulfolobus acidocaldarius*. The results demonstrate that in both heme groups the structural properties depart from those of other aa_3 oxidases. In the fully oxidized form, the formyl stretching vibration of heme *a* is observed at 1656 cm^{-1}, approximately 7 cm^{-1} higher in frequency than found for beef heart cytochrome *c* oxidase. The frequency of this vibration shows a slight upshift to 1657 cm^{-1} in the fully reduced form. Thus, the formyl group of the heme *a* seems not to be involved in a significant hydrogen bond, in sharp contrast to both beef heart cytochrome *c* oxidase and other quinol-oxidizing enzymes. For heme a_3, two conformers were detected in the fully oxidized state. The observation of two ν_3 modes at 1482 and 1490 cm^{-1} indicate the coexistence of both the normal six-coordinated high spin and a new five-coordinated high spin configuration. Both heme a_3 species exhibit different formyl stretching vibrations at 1673 and 1666 cm^{-1}, respectively. In the fully reduced enzyme, the identification of two heme a_3 conformers is not unambiguous.

Sulfolobus acidocaldarius is a member of a group of aerobic archaebacteria living at high temperature in low pH media (Brock et al., 1972). Its ability to survive under these conditions makes it an interesting organism from the standpoint of cellular energetics. Previous studies have determined that it creates a large proton gradient across the plasma membrane, and this feature is coupled to proton pumps, driven by respiratory activity (Moll & Schäfer, 1988). These aspects of energy transduction are of current interest, and more experimental data on the structural and mechanistic details of the various proton pumps are required for a proper understanding of their functioning (Babcock & Wikström, 1992; Malmström, 1990; Wikström et al., 1981).

The terminal oxidase from *S. acidocaldarius* (SOX)[1] was first isolated as a single-subunit cytochrome aa_3 with an apparent molecular mass of 38–40 kDa (Anemüller & Schäfer, 1989). This polypeptide was later identified as the product of the SOX-B gene in an operon coding for a larger electron transport complex also containing the gene (SOX-C) for an additional heme protein (Lübben et al., 1992). According to its amino acid sequence, the aa_3 polypeptide has a true molecular mass of 57.9 kDa and, like well-characterized terminal oxidases, exhibits 12 putative membrane-spanning helices. The single polypeptide oxidase shows similarity with eukaryotic cytochrome oxidases in terms of electronic absorption spectra (Anemüller & Schäfer, 1990). The redox centers have been proposed to consist of heme *a* and heme a_3 as well as one copper ion, identified as Cu_B of the binuclear center. The hemes in SOX show midpoint potentials similar to those of the corresponding hemes in beef heart cytochrome *c* oxidase (BOX). However, the former protein oxidizes reduced caldariella quinone, a thiophenobenzoquinone analog of ubiquinone found in the membranes of *Sulfolobus* (DeRosa et al., 1977), but it shows no activity with ferrocytochrome *c*. Known quinol oxidases differ from aa_3-type oxidases most prominently in that the former lack Cu_A and appear to oxidize quinols directly with heme *a* (Gennis, 1991; Lauraeus et al., 1991; Puustinen et al., 1991; Anemüller et al., 1992).

Resonance Raman (RR) spectroscopy has achieved considerable success in the elucidation of many important aspects of heme protein structure and function (Spiro & Li, 1988). The responsive behavior of certain bands in the spectral region between 1450 and 1650 cm^{-1} (the marker band region) to changes in heme oxidation, spin, and coordination state has been shown to be extremely reliable, and its understanding has reached a highly predictive level (Kitagawa & Ozaki, 1987; Spiro & Li, 1988). Applications of RR spectroscopy to the study of cytochrome oxidases have revealed that heme *a* is six-coordinate, low spin (6cLS) in both oxidation states and that heme a_3 is six-coordinate, high spin (6cHS) in the oxidized state and five-coordinate, high spin (5cHS) in the reduced state (Callahan & Babcock, 1981; Choi et al., 1983; Ogura et al., 1985; Ching et al., 1985; Babcock, 1988). This coordination change is related to the function of heme a_3 in BOX, which is to bind O_2 and allow a controlled reduction.

The present study extends our previous RR observations (Hildebrandt et al., 1991), employing higher resolution and an improved sample preparation which allows more detailed examination of the heme organization in this novel oxidase. We investigate the fully oxidized (SOX_{ox}) and fully reduced (SOX_{red}) forms of the terminal oxidase in the marker band

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[1] Abbreviations: BOX, beef heart cytochrome *c* oxidase; SOX, *Sulfolobus acidocaldarius* cytochrome aa_3; the subscripts "ox" and "red" denote the fully oxidized and fully reduced forms, respectively; RR, resonance Raman; 6cLS, six-coordinated low spin; 6cHS, six-coordinated high spin; 5cHS, five-coordinated high spin.

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region (1440–1700 cm^{-1}), which allows observation of the spin state marker bands as well as of substituent stretching vibrations.

MATERIALS AND METHODS

Biochemical Isolation and Sample Preparation. The terminal oxidase from *S. acidocaldarius* was isolated as described previously, with the following modifications. The chaotropic extraction of membranes was replaced by extraction with 30 mM sodium pyrophosphate in 50 mM malate buffer, pH 5.5; thereby the overall yield and spectral purity of the final product was largely enhanced. After hydroxyapatite chromatography, the aa_3-containing fractions were concentrated on an Amicon PM-10 membrane and rechromatographed. Thereby, the overall yield and spectral purity of the product were significantly improved. The final preparation containing only the single-subunit form of the terminal oxidase from *S. acidocaldarius* was extensively dialyzed against a 1000-fold volume of 10 mM potassium phosphate, 10 mM EDTA, and 0.05% sarcosyl and reconcentrated thereafter. The optical absorption maxima were at 421 and 597 mm in the oxidized and at 439 and 601 nm in the reduced state (Anemüller & Schäfer, 1990). The metal content was determined by atomic absorption spectroscopy. The resulting preparations contained 1.6–2.1 mg of protein/mL with 1.9–2.3 mol of Fe/mol of aa_3, and 0.45–0.5 mol of Cu/mol of Fe. *Sulfolobus* oxidase prepared as described has high quinol oxidase activity and does not contain Cu_A as also documented by the EPR spectra (Anemüller et al., 1992). Beef heart mitochondria were prepared according to the procedure described in Heibel et al. (1993). All samples were shock frozen in liquid nitrogen and stored at –60 °C. The thawed samples were diluted with buffer to an optical density of 1.0 at 413.1 nm. Reduction of the oxidase was achieved using a minimal amount of solid sodium dithionite.

Spectral Acquisition. Laser excitation at 413.1 nm was provided by a Kr^+ laser (Spectra Physics), operated at ca. 20 mW. The scattered light was collected at 90° from a rotating quartz cuvette and focused onto the entrance slit of a Spex 1404 double monochromator equipped with a photon counting system, including a cooled photomultiplier tube (RC 31034/76). The spectra were collected with a step size of 0.2 cm^{-1} with an acquisition time of 0.5 s at each step. Spectral resolution was 3 cm^{-1}. Photoreduction was not observed under our conditions. The intrinsic fluorescence of *Sulfolobus* aa_3 which obscured much of the spectral information in previous studies was reduced by approximately 75% through the modified isolation procedure. All spectra were recorded at room temperature.

Data Handling. Spectra displayed are the sum of 8–12 scans, each scan requiring 20 min. The fluorescence background was subtracted using polynomial subtraction. For the summed spectra shown in this paper we have employed a filter which lowered the high-frequency noise level to the extent that subtraction of the filtered spectra from the original spectra resulted in evenly distributed noise with an amplitude of 0.5–1.0% of the average number of counts of the original spectra. Thus, the filtering procedure does not add or remove any spectral features from the spectra so treated.

Band Fitting. Spectra were analyzed with an interactive band fitting program based on a Marquardt algorithm using Lorentzian lines to fit the Raman bands. Other spectral lineshapes (Gaussian or Voigt functions) were not suitable for the spectral simulation, as previously noted in other studies of the RR spectra of heme proteins (Hildebrandt & Stockburger, 1989; Hildebrandt et al., 1992; Heibel et al., 1993). The fit was achieved by progressively lowering the damping constants restraining the band parameters, until the difference between the measured and simulated spectra was within the noise level of the measured spectrum. This led to average standard deviations in SOX spectra of 0.8 cm^{-1} in frequency and 3.0 cm^{-1} in bandwidth, except for those bands in more crowded spectral regions (1470–1510 cm^{-1} in SOX_{ox}) for which the respective standard deviations in the band parameters for the latter bands were 1.5 and 6.0 cm^{-1}. Standard deviations for BOX spectral parameters were smaller by a factor of approximately 2.5.

RESULTS AND DISCUSSION

The fully oxidized and fully reduced SOX were measured with 413-nm excitation in the marker band region (1450–1700 cm^{-1}). Upon Soret band excitation, this region displays the heme *a* and heme a_3 skeletal modes of A_{1g} (ν_2, ν_3,), B_{1g} (ν_{10}, ν_{11}), and B_{2g} (ν_{28}) symmetry [mode notation according to Abe et al. (1978)], which have been shown to be reliable indicators of core size, spin, oxidation, and ligation state of heme proteins in general and of cytochrome oxidases in particular (Babcock, 1988). The E_u (ν_{37}, ν_{38}) modes are also enhanced with this excitation wavelength and reflect the asymmetry of the heme substitution pattern and electronic distribution (Choi et al., 1983). In addition, this spectral region displays bands containing a dominant contribution of the porphyrin *a* peripheral substituent (formyl, vinyl) stretching vibrations. These bands are expected to reflect sensitively the immediate environment of the hemes, since they are most likely in direct contact with amino acid residues of the heme pocket. With 413-nm excitation, the resonance Raman (RR) spectra of aa_3-type oxidases include contributions from both heme *a* and heme a_3, leading to a large number of overlapping bands in this region. This is reflected by the broad and asymmetric peaks in the spectrum of SOX_{ox} shown in Figure 1A. Thus, it is helpful to compare this spectrum with that of BOX_{ox} (Figure 1B), for which a detailed vibrational analysis has been carried out (Heibel et al., 1993).

The Fully Oxidized State. The RR spectrum of SOX_{ox} in Figure 1 is similar to that previously reported (Hildebrandt et al., 1991), although there are some differences which may be partly due to the improved isolation procedure in the present work. For example, the intensity of the 1589-cm^{-1} peak is higher than in the previously reported spectrum. In addition, a higher structural differentiation of the lower intensity peaks below 1560 cm^{-1} is also discernable in the present spectrum due to the significantly improved spectral resolution.

A comparison of SOX_{ox} and BOX_{ox} in Figure 1 shows a number of important differences. There are large intensity changes in all spectral regions, most obviously in the 1650- and 1570-cm^{-1} regions. The spectral region between 1460 and 1510 cm^{-1} is clearly more crowded in the bacterial oxidase than in the beef heart enzyme. The highest frequency region, above 1645 cm^{-1}, is very different for the bacterial enzyme. We have tried to ascribe these differences to the individual hemes in SOX_{ox} by employing a band fitting analysis to deconvolute the bands in these spectra.

A comparison of the 1450–1560-cm^{-1} region for BOX_{ox} and SOX_{ox} is made in Figure 2. This region contains the ν_3 marker band which is relatively well isolated from interfering modes and is commonly used to determine the spin and coordination states of hemes in heme proteins. The RR bands

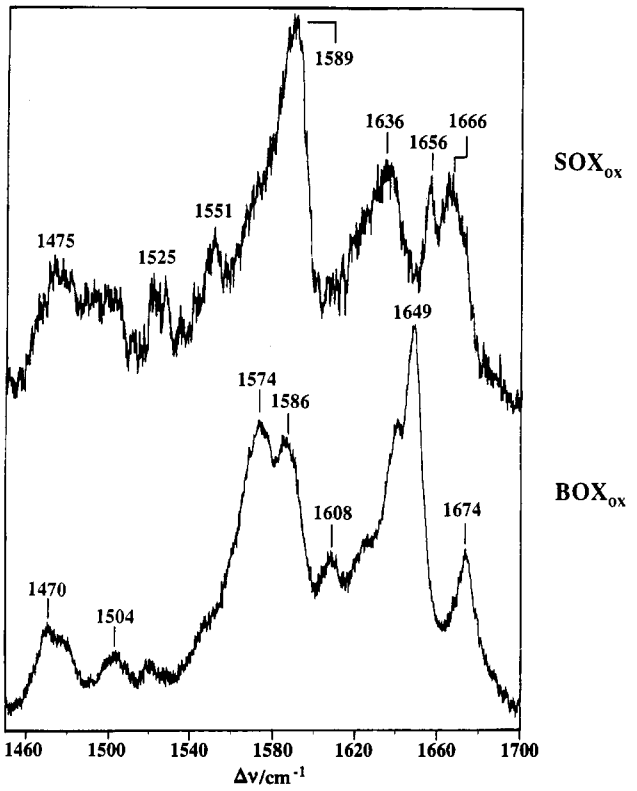

FIGURE 1: Resonance Raman spectra of (A) SOX_{ox} and (B) BOX_{ox} in the spectral region from 1450 to 1700 cm^{-1} obtained with 413-nm excitation.

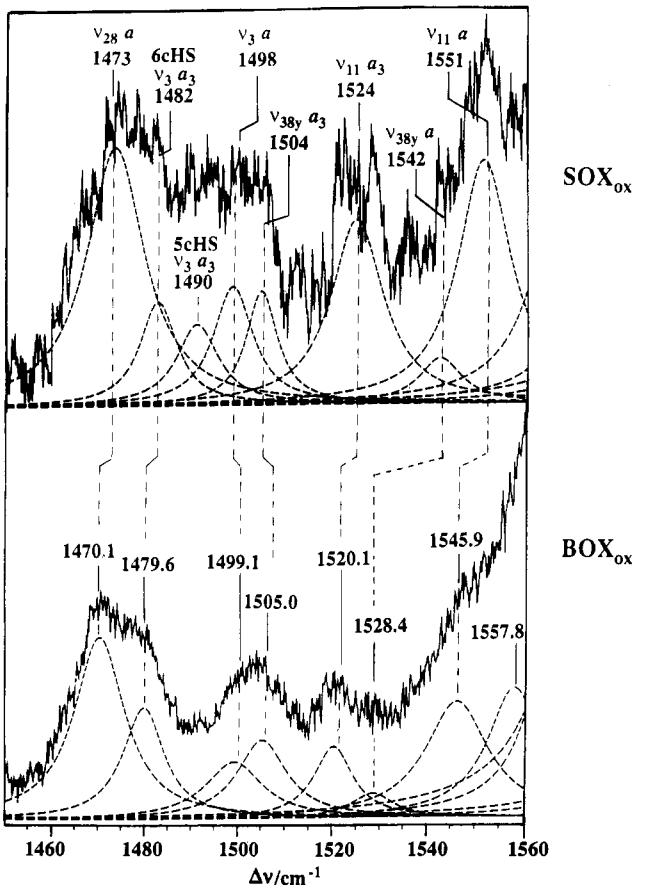

FIGURE 2: Resonance Raman spectra of (A) SOX_{ox} and (B) BOX_{ox} in the spectral region from 1450 to 1560 cm^{-1}, together with the component Lorentz bands obtained from band fitting.

for SOX_{ox} are assigned in analogy to BOX_{ox}, as far as possible (Heibel et al., 1993; Babcock, 1988; Table I). There are SOX bands corresponding to each BOX band in this spectral region, although the most remarkable difference between the two spectra is the observation of a peak located at ~1490 cm^{-1} in SOX_{ox} which has no counterpart in the RR spectrum of BOX_{ox}. It is evident that this spectral region cannot be fitted by allowing the four existing BOX_{ox} bands to broaden and fill the "valley" which is found at this position in BOX_{ox}. The 1490-cm^{-1} band occurs at a characteristic frequency for the ν_3 band of a 5cHS heme (Babcock, 1988). Consequently, we conclude that, in the *Sulfolobus* enzyme, one of the heme groups exists in a coordination equilibrium between a 5c and 6c state. In BOX_{ox} and other cytochrome *c* oxidases, heme *a* is ligated by two histidines which, by virtue of their strong ligand field character, yield a 6cLS configuration. In heme a_3, a histidine and a weak field ligand, as yet unidentified, coordinate the central iron, leading to a 6cHS configuration in BOX. The evidence for a 5cHS population in SOX_{ox} can be interpreted as an even more weakly binding heme a_3 axial ligand.

These findings imply that in the entire spectral region we must take into account three different species: the 6cLS heme *a* and both the 6cHS and 5cHS configurations of heme a_3. This imposes serious difficulties on the vibrational analysis of the RR spectrum, in particular in the region between 1560 and 1630 cm^{-1} which is expected to include up to 14 bands. In this region, a band fitting analysis cannot yield unambiguous results. Nevertheless, a qualitative examination of this region (Figure 1) also supports the conclusion of coexisting 5cHS and 6cHS states of heme a_3: the distinct peak at 1574 cm^{-1} in BOX_{ox}, which is due to the ν_2 peak of heme a_3 (6cHS), is replaced in SOX_{ox} by a much weaker but significantly broader shoulder of the 1589-cm^{-1} peak (ν_2 of heme *a*) which can be readily understood in terms of lowering of the heme a_3 6cHS population. In addition, the intensity increase between 1620 and 1640 cm^{-1} indicates the population of the 5cHS configuration of heme a_3 (ν_{10}).

Table I: Assignments for Beef Heart and *Sulfolobus* Oxidases[a]

	beef heart		*Sulfolobus acidocaldarius*	
	frequency	width	frequency	width
heme a, oxidized				
C=O (6cLS)	1649.0	9.2	1656	8
ν_3 (6cLS)	1499.1	14.8	1498	10
ν_{28} (6cLS)	1470.1	13.1	1473	15
heme a_3, oxidized				
C=O (6cHS)	1673.6	13.2	1673	7
C=O (5cHS)			1666	12
ν_3 (5cHS)			1490	11
ν_3 (6cHS)	1479.6	10.8	1482	10
heme a, reduced				
C=O (6cLS)	1609.2	12.6	1657	10
ν_3 (6cLS)	1490.8	6.9	1494	13
ν_{28} (6cLS)	1466.1	16.0	1462	17
heme a_3, reduced				
C=O (HS)	1664.7	9.4	1668	(9)
C=O (5cHS)			(1672)	(10)
ν_3 (HS)	1473.6	6.9	1476	11
ν_3 (5cHS)			(1469)	(13)

[a] All numbers are given in cm^{-1}.

A reliable band fitting of the RR spectrum of SOX_{ox} is also possible above ~1630 cm^{-1}. This region, which includes the stretching vibrations of the formyl substituents, is shown in Figure 3 for both SOX_{ox} and BOX_{ox}. The spectral region above 1650 cm^{-1} shows the presence of three bands in SOX_{ox}. The spectrum cannot be satisfactorily fitted without including the highest frequency band at 1673 cm^{-1}. We assign all three of these bands as formyl stretching vibrations (Table I). The

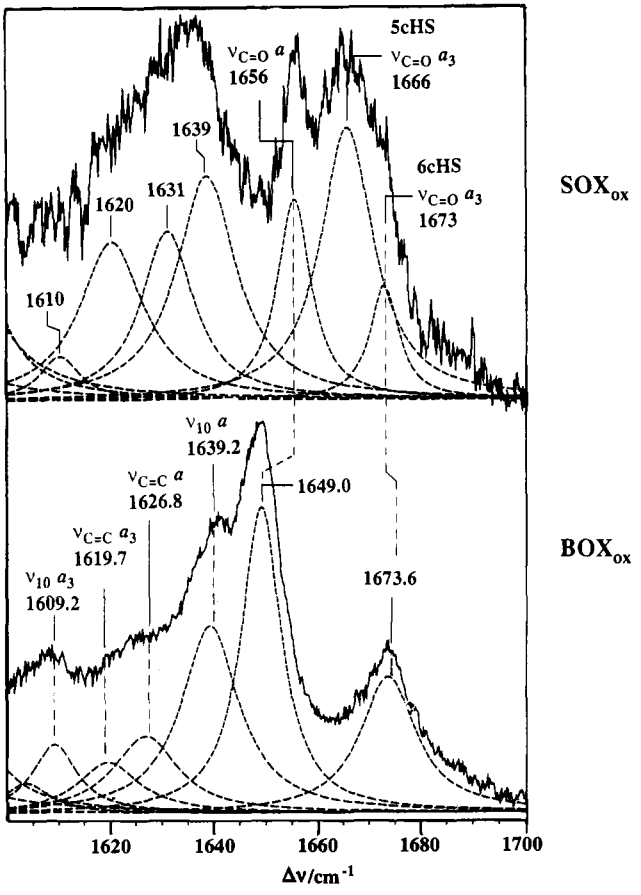

FIGURE 3: Resonance Raman spectra of (A) SOX_{ox} and (B) BOX_{ox} in the spectral region from 1600 to 1700 cm^{-1}, together with the component Lorentz bands obtained from band fitting.

behavior of porphyrin *a* formyl stretching vibrations has been the object of a great deal of experimental effort (Babcock, 1988; Han et al., 1991). The heme *a* formyl stretch in BOX_{ox} is located at 1649 cm^{-1}, approximately 6 cm^{-1} lower than that of a 6cLS model compound in aqueous medium (Han et al., 1991; Babcock, 1988). This downshift is attributed to strong hydrogen bonding within the protein. We assign the sharp peak at 1655 cm^{-1} of SOX_{ox} to a heme *a* formyl stretching vibration which is notably less hydrogen bonded than that of BOX_{ox}, similar to an aqueous environment.

On the other hand, heme a_3 of BOX_{ox} displays a formyl vibration at 1674 cm^{-1}. It has yet to be shown that the formyl vibration can be used to predict the coordination state in porphyrin *a* model compounds. Thus, although the 5cHS porphyrin a^{3+} Cl^-/CH_2Cl_2 and the 6cHS porphyrin a^{3+} $(DMSO)_2/DMSO$ show formyl stretches at 1676 and 1672 cm^{-1}, respectively (Callahan & Babcock, 1981), the different dielectric properties of methylene chloride and dimethyl sulfoxide may be responsible for this frequency shift, analogous to the work of Koyama et al. (1986) on metallochlorins. We are therefore unable to make a conclusive assignment for the bands at 1666 and 1673 cm^{-1} with respect to the two coordination states of heme a_3, but it may be expected that the 5cHS heme a_3 will show a formyl stretching vibration distinct from that of the 6cHS configuration. It seems reasonable to assume that the 1673-cm^{-1} band, being closer in frequency to the BOX_{ox} heme a_3 formyl stretch, corresponds to a formyl in a similar position as in standard 6cHS heme a_3 configuration found in BOX_{ox} (Heibel et al., 1993; Babcock, 1988) as well as the quinol oxidase from *Bacillus subtilis* (Lauraeus et al., 1992). Then the band at 1666 cm^{-1} may

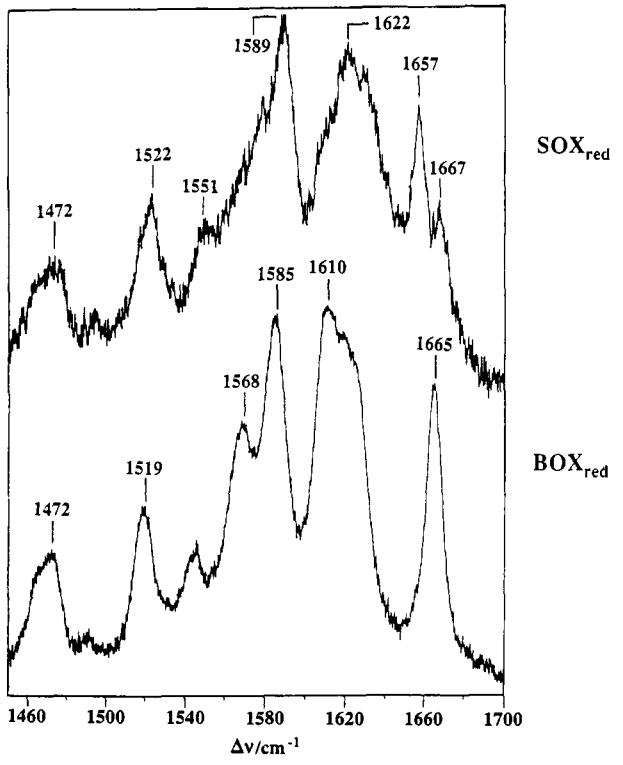

FIGURE 4: Resonance Raman spectra of (A) SOX_{red} and (B) BOX_{red} in the spectral region from 1450 to 1700 cm^{-1} obtained with 413-nm excitation.

represent a formyl on a 5cHS heme a_3. However, the relative intensity of RR bands cannot be regarded as a reliable guide to their relative populations.

The vinyl stretching region (1610–1640 cm^{-1}) of SOX_{ox} is clearly broader, relatively more intense, and less structured than in BOX_{ox}. This can be ascribed to the additional contributions from the 5cHS species, so that the number of bands expected in this region is six instead of four in BOX_{ox} (ν_{10} and $\nu_{C=C}$ for each species). This spectral region has been fitted with the minimum number of components required to satisfactorily simulate the spectrum, implying that such components do not reflect real bands in each case. While the components at ~1610 and 1640 cm^{-1} agree quite well with ν_{10} bands of heme a_3 and heme *a* of BOX_{ox}, respectively, the components at ~1620 and 1630 cm^{-1} include all three vinyl stretching vibrations and the ν_{10} mode of the 5cHS heme a_3.

The Fully Reduced State. The RR spectrum of SOX_{red} is shown in the marker band region (1440–1700 cm^{-1}) in Figure 4A, again in comparison with the spectrum of the fully reduced beef heart oxidase, which has been analyzed in detail elsewhere (Heibel et al., 1993). In the mammalian enzyme, the spin configuration is preserved upon reduction for both hemes, although, unfortunately, the marker band frequencies of 6cHS and 5cHS ferrous porphyrins are very similar, so that a distinction between heme a_3 coordination states solely from RR spectra is not possible. Mössbauer spectroscopy (Kent et al., 1982; Wang et al., 1988) and X-ray absorption (Scott, 1989) have revealed that heme a_3 in BOX_{red} has similar spectral characteristics as deoxymyoglobin, possessing a 5cHS configuration and being able to bind O_2.

The RR spectrum of SOX_{red} displayed in Figure 4A shows some differences compared to that reported previously (Hildebrandt et al., 1991). The intensity of the sharp peak at 1657 cm^{-1} as well as the intensity of the ν_2 envelope at 1589 cm^{-1} relative to the ν_{10}–vinyl stretching region (1610–1640 cm^{-1}) is much higher in the present spectrum.

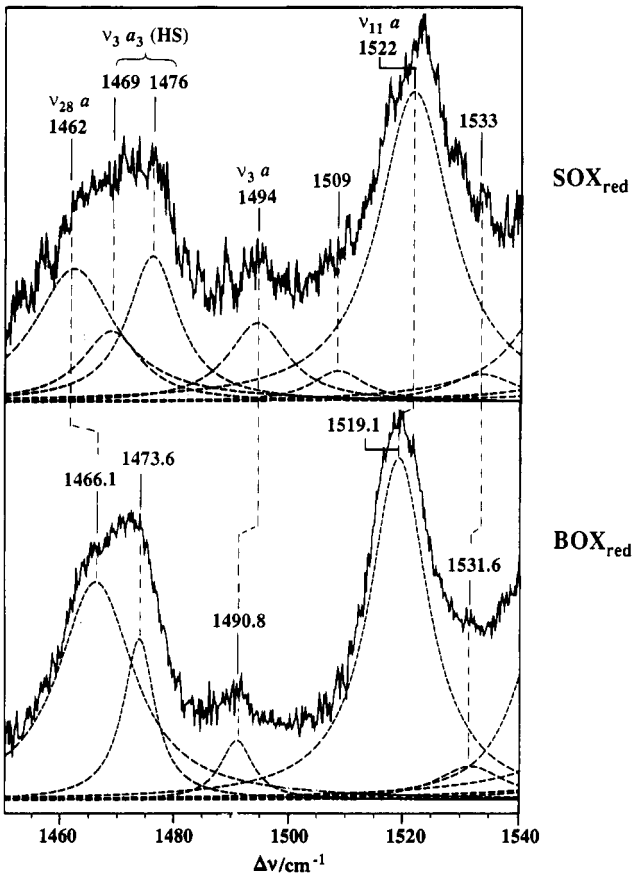

FIGURE 5: Resonance Raman spectra of (A) SOX_{red} and (B) BOX_{red} in the spectral region from 1450 to 1540 cm^{-1}, together with the component Lorentz bands obtained from band fitting.

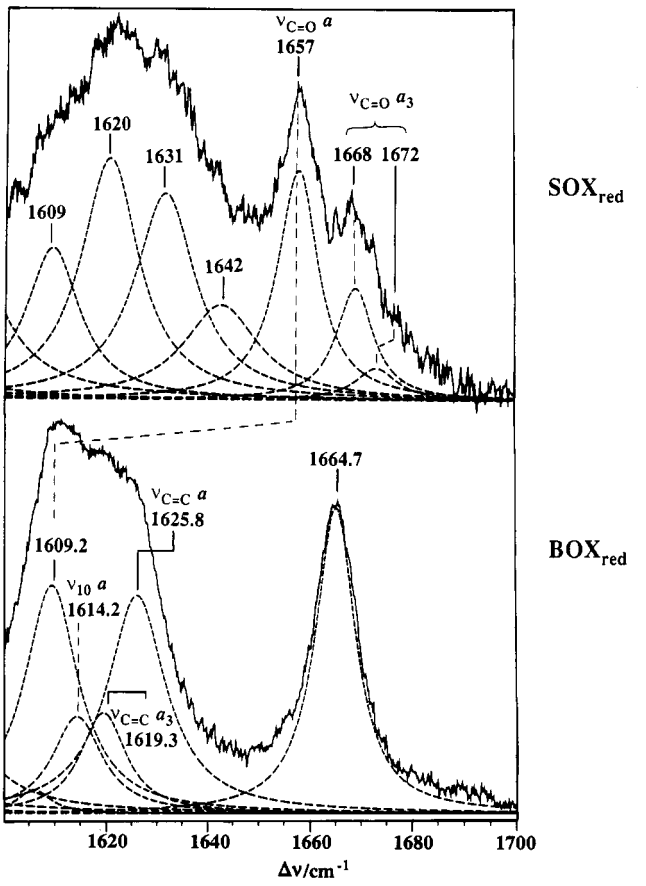

FIGURE 6: Resonance Raman spectra of (A) SOX_{red} and (B) BOX_{red} in the spectral region from 1600 to 1700 cm^{-1}, together with the component Lorentz bands obtained from band fitting.

The inspection of BOX_{red} and SOX_{red} reveals obvious differences (Figure 4). Most of the spectral features are broader in SOX_{red}, and several peaks are significantly shifted. The peak at 1568 cm^{-1}, which in BOX_{red} constituted an intense doublet with the 1588-cm^{-1} peak, has lost intensity and is hidden as a broad and poorly resolved shoulder. Since in BOX_{red} the main contribution to the 1570-cm^{-1} peak comes from the ν_2 mode of heme a_3, we conclude that in SOX_{red} the spectral differences are associated with the HS heme a_3. The lowered intensity of ~1570 cm^{-1} cannot be attributed to more unfavorable resonance conditions of heme a_3, since other modes, such as ν_3, are not weaker than in BOX_{red} (see below). Instead, these observations are reminiscent of the fully oxidized state where the broadening and intensity decrease of the 1570-cm^{-1} peak has been ascribed to the overlapping contributions from the 5cHS and 6cHS configurations. Thus the question arises if, in analogy to the oxidized state, there is also an equilibrium between two HS forms in SOX_{red}.

In fact, the analysis of the ν_3 band region which is presented in Figure 5A in an expanded view may support this idea. While the ν_3 mode of the 6cLS heme *a* is expected at ~1490 cm^{-1} and can readily be assigned to the weak band at 1494 cm^{-1}, the corresponding mode of a 5cHS configuration is expected between 1470 and 1475 cm^{-1}. In this part of the spectrum, the asymmetric peak at ~1470 cm^{-1} is similarly shaped but much broader than in BOX_{red}. In the latter oxidase, this peak includes the mode ν_{28} (heme *a*) and ν_3 (heme a_3) at ~1466 and 1474 cm^{-1}, respectively (Table I). In SOX_{red} a satisfactory fit is also achieved with two components which are, however, much broader than in BOX_{red}. Thus it is possible that there is a third component which would correspond to a second HS form of heme a_3. The formyl and vinyl stretching regions (1600–1700 cm^{-1}) for reduced BOX_{red} and SOX_{red} are shown in Figure 6, revealing the most notable differences between the mammalian and *Sulfolobus* oxidases. The BOX_{red} heme *a* formyl stretching vibration is assigned to a band at ca. 1610 cm^{-1} (Babcock, 1988). We observe lower intensity in this region in the SOX_{red} spectrum. We previously demonstrated that intensity at ~1610 cm^{-1} in SOX_{red} is attributable to a heme a_3 vibration (ν_{10}), because of lowered intensity upon CO binding in the reduced state (Hildebrandt et al., 1991). Thus, we conclude that, of the bands observed above 1650 cm^{-1} in SOX, one should be assigned to the formyl stretching of heme *a*. This difference is a drastic departure from the classical cytochrome oxidase architecture (Babcock, 1988).

The peak at ~1670 cm^{-1} is within the region expected for the formyl stretching of heme a_3, with the carbonyl group being in an non-hydrogen-bonding environment. Consequently, the remaining band in this region at 1657 cm^{-1} is ascribed to the formyl stretch of heme *a*. This frequency is about 12 cm^{-1} higher than those of 6cLS model compounds measured in nonaqueous media [1645 cm^{-1} in CH_2Cl_2 (Babcock, 1988); 1644 cm^{-1} in DMSO (Choi et al., 1983)]. This implies that a non-hydrogen-bonding environment is not the only origin for this unusually high frequency. In addition, it may be that geometric distortion, i.e., an out-of-plane tilting of this substituent, weakens the coupling with the π-electron system of the porphyrin, bringing the carbonyl stretching frequency closer to that of an isolated aldehyde group (Dollish et al., 1974).

Also the band fitting analysis of the 1670-cm^{-1} envelope does not provide an unambiguous conclusion whether or not there are two HS forms in the reduced heme a_3. As in the

ν_3 band region, the peak is broad and asymmetric but a fit with only one component gave similarly good standard deviations as the two-component fit which is shown in Figure 6.

As emphasized for the oxidized state, the possible presence of a new coordination state for heme a_3 renders the band fitting of the 1600–1650-cm^{-1} spectral region of Figure 6 ambiguous. The band fitting of the vinyl stretching region in SOX_{red} represents a minimum number of bands required to fit the spectrum. We note the extreme width of these bands and also that the spectral intensity at ca. 1642 cm1^{-1} is highly unusual, since heme skeletal vibrations are not found above 1640 cm^{-1}. This band is probably due to a vinyl stretching vibration.

Structural Differences Compared to Other Oxidases. In the oxidized *Sulfolobus* enzyme, the frequency upshift of the formyl stretching mode of heme *a* compared to the bovine oxidase implies weaker hydrogen-bonding interactions of this substituent. This finding is in line with the blue-shift of the α-absorption band by 4 nm to 597 nm (Babcock & Callahan, 1983). The *Sulfolobus* enzyme also differs from another quinol oxidase from *B. subtilis* which reveals a formyl stretching frequency similar to that of BOX_{ox} (Lauraeus et al., 1992). On the other hand, a similarly high frequency as in SOX_{ox} was also observed for the formyl stretching in wheat germ cytochrome oxidase (de Paula et al., 1990) which, however, shifts down to 1610 cm^{-1} upon reduction as observed for all oxidases studied so far, including the quinol oxidase from *B. subtilis* (Babcock, 1988; Lauraeus et al., 1992). This is in sharp contrast to SOX_{red}, where the stretching mode remains at the same position upon reduction. This implies that the redox-linked change of the hydrogen-bonding interactions of this substituent is not an unique structural property of the oxidase superfamily.

On the other hand, the present data do not provide evidence for significant structural differences of the porphyrin skeleton of heme *a* itself. The 1589-cm^{-1} peak of SOX_{ox} is at a slightly higher frequency than the corresponding peak of BOX_{ox} (Figure 1), which may be due to an upshift of the mode ν_2 of heme *a*. This as well as the upshift of ν_{11} (Figure 2) may reflect differences in the electronic coupling between the porphyrin and the conjugated substituents (Choi et al., 1983; Willems & Bocian, 1984; Heibel et al., 1993). Presumably, these differences are not related to the unique hydrogen-bonding interactions of the formyl group since a similarly high frequency of the ν_2 peak was also noted for quinol oxidase from *B. subtilis* (Laureaus et al., 1992).

The coexistence of 5cHS and 6cHS forms of oxidized heme a_3 is the most pronounced difference compared to heme a_3 of other cytochrome *c* oxidases as well as of the quinol oxidases from *B. subtilis*. This finding suggests that the interaction with the sixth ligand is weaker in SOX than in BOX. In this context, we wish to mention the unusual EPR spectroscopic behavior of heme a_3 of SOX_{ox} (Anemüller & Schäfer, 1990; Anemüller et al., 1992). A strong signal at $g = 6$ was found which in BOX is only observed after breaking the antiferromagnetic coupling between heme a_3 and Cu_B by partial reduction. A partial reduction of the heme groups in SOX_{ox}, however, can be ruled out by the RR spectra. In particular, the ν_4-band region does not reveal a peak or a shoulder at $\sim$1360 cm^{-1}. A different EPR response was observed upon azide binding. A low spin signal at $g_z = 2.9$ as noted in BOX_{ox} was not found for the *Sulfolobus* enzyme which instead reveals a broadening of the $g = 6$ signal. Thus, these EPR data point to some structural anomalies of the heme a_3–Cu_B binuclear center which may be related to the distortion of the coordination sphere of the heme a_3 as revealed by the RR spectra. However, it should be noted that the distribution between the 5cHS and 6cHS forms is not necessarily the same in the EPR and RR experiments since both configurations may constitute a temperature-dependent equilibrium.

Both HS species exhibit different formyl stretching frequencies, which either reflects an intrinsic dependence of this mode on the coordination state (in analogy to the porphyrin marker bands) or, more likely, subtle differences in the interactions with the protein environment. This latter interpretation implies that both HS species differ not only in the proximity of the sixth ligand with respect to the heme iron but also in molecular structure in the vicinity of the formyl substituent. Following this idea, the two HS forms would reflect conformers rather than a simple coordination equilibrium. This would be the most plausible explanation if there were two heme a_3 HS species in the fully reduced state as well.

Finally, we wish to emphasize that the coexistence of two HS forms does not result from a partial denaturation of the enzyme during isolation and purification. It has been demonstrated that the *Sulfolobus* oxidase is constitutively active, indicating its functional and structural integrity (Gleissner et al., 1992).

The striking differences of the heme structures between SOX and BOX cannot be attributed to the different physiological reductants, i.e., caldariella quinone and cytochrome *c*, respectively. The far reaching similarities of the quinol oxidase from *B. subtilis* and the mammalian enzyme indicate that the specificity on the binding site does not affect the structure of the heme pocket. The same arguments also rule out that the lack of Cu_A in both quinol oxidases influences the active sites (Laureaus et al., 1992). It may be that the specific heme pocket structures in SOX may reflect the structural adaption of this enzyme to the extreme living conditions of this archaebacterium.

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