Volume 283, number 1, 131–134 FEBS 09736 May 1991

ADONIS 001457939100445N

Resonance Raman study of cytochrome aa_3 from *Sulfolobus acidocaldarius*

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Received 14 March 1991

The single subunit terminal oxidase of *Sulfolobus acidocaldarius*, cytochrome aa_3, was studied by resonance Raman spectroscopy. Results on the fully oxidized, the fully reduced, and the reduced carbon monoxide complex are reported and compared with those of eucaryotic cytochrome oxidase. It is shown that in both redox states the hemes *a* and a_3 are in the six-coordinated low-spin and six-coordinated high-spin configuration, respectively. The resonance Raman spectra reveal far-reaching similarities of this archaebacterial with mammalian or plant enzymes except for the reduced form of heme *a*. The formyl substituent of this heme appears above 1640 cm^{-1}, ruling out significant hydrogen bonding interactions which is in sharp contrast to beef heart cytochrome oxidase. In addition, frequency upshifts of the marker bands ν_4 and ν_2 are noted indicating differences in the electron density distribution within the molecular orbitals of the porphyrin.

Cytochrome aa_3; *Sulfolobus acidocaldarius*; Archaebacteria; Resonance Raman spectroscopy

1. INTRODUCTION

Sulfolobus acidocaldarius is a representative of a class of aerobic archaebacteria living in highly acidic media and at extreme temperature [1]. This thermophilic organism is capable of maintaining a large proton gradient across the plasma membrane which is thought to be coupled to a respiration-driven proton pump [2]. Both the phylogenetic relation of *S. acidocaldarius* to eucaryotes and its ability to adapt to extreme conditions stimulates structural and functional investigations of its respiratory chain [2–8]. Recently, Anemüller and Schäfer succeeded in isolating a terminal oxidase which consists of a single polypeptide subunit with a molecular mass of 38–40 kDa [4,5]. Its catalytic activity towards reduced cytochrome *c* was found to be negligible but, instead, it can effectively oxidize caldariella quinone, the ubiquinone analog in the membrane of *S. acidocaldarius*. Based on electron absorption and EPR studies some similarities with eucaryotic cytochrome oxidases were revealed [5]. Evidence was provided for a heme *a* and a heme a_3 as well as for one of the two Cu-ions which constitute the redox centers in this novel oxidase. Also the midpoint redox potentials of both heme groups closely resemble those of mammalian cytochrome oxidase.

In the present work, the structural studies of this oxidase from *S. acidocaldarius* were continued by employing resonance Raman (RR) spectroscopy. This technique selectively probes the heme groups and may provide valuable information about their conformations and interactions with the immediate protein environment [9]. Our findings will be compared with previous results on mammalian and plant cytochrome oxidase and structural similarities and differences will be discussed.

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Abbreviations: RR, resonance Raman; aa_3, cytochrome aa_3 from *Sulfolobus acidocaldarius*; 6cLS, six-coordinated low-spin; 6cHS, six-coordinated high-spin

2. MATERIALS AND METHODS

Growth of bacteria and protein isolation were described elsewhere [3–5]. For RR experiments, the protein was solubilized in 0.8 M phosphate buffer (pH 7.4) and 0.05% sarcosyl with an optical density of about 2.5 at 420 nm. Complete reduction of the oxidase was achieved by adding a few grains of sodium dithionite. The carbon monoxide complex was prepared by flowing a stream of CO on the surface of the reduced protein solution.

RR spectra were excited with the 413 nm line of a Kr^+-laser and measured with an optical multichannel system including a Spex Triplemate equipped with an intensified photodiode array (Spectroscopy Instruments). The spectral resolution was ~5 cm^{-1} and the resolution per diode ~1 cm^{-1}. The sample was deposited in rotating cell in order to avoid photoreduction or laser-induced damage of the protein. The laser power at the sample was about 15 mW, focused onto the cell by a 8 cm lens. The total accumulation and averaging time was about 5 h. No time-dependent spectral changes were noted during the RR experiments. The quality of the RR spectra suffered strongly from an intense and broad fluorescence with a maximum at 480 nm. Its intensity was nearly two orders of magnitude higher than the

strongest RR band, accounting for the relatively poor S/N ratio even after prolonged averaging. It should be noted that the fluorescence could not be removed by using other detergents for protein solubilization. In the RR spectra which were displayed in this paper the background was subtracted using a third-order polynomial.

3. RESULTS

Fig. 1 shows the RR spectrum of the fully oxidized cytochrome aa_3 ($a^{3+}a_3^{3+}$). The 413 nm excitation line is in resonance with the Soret transitions of both heme chromophores. The dominant peak in the RR spectrum at 1371 cm^{-1} is readily assigned to the porphyrin mode ν_4 (following the notation by Abe et al. [10]). The observed frequency is typical for a ferric heme [11,12] implying that it includes contributions from both a^{3+} and a_3^{3+}. The spectral region above 1500 cm^{-1} is displayed on an extended view in Fig. 2A. This part of the RR spectrum is of particular importance since it includes bands for which valuable empirical correlations with structural parameters of the heme and its interactions with the protein environment are well established. The frequencies of some of these bands are diagnostic for the spin and coordination state [11,12]. The strongest marker band at 413 nm excitation is ν_2 for which a ferric heme in the six-coordinated low-spin (6cLS) configuration is expected at ~1585 cm^{-1}. A band close to this position (i.e. at 1589 cm^{-1}) is apparently the major contributor to the broad peak centered at ~1580 cm^{-1} (Fig. 2A) suggesting that one of the hemes, i.e. heme a, is in the 6cLS state. A second component of this peak can be identified at ~1575 cm^{-1}, indicating a high-spin heme and, hence, it is attributed to heme a_3. The good agreement with the ν_2 frequency of heme a_3 of mammalian or plant cytochrome oxidase (Table I; [13,14]) points to a 6-fold coordination of the HS form (6cHS).

No porphyrin fundamentals are expected at frequencies above 1645 cm^{-1}. Thus, the broad peak which is apparently composed of two bands at ~1658 and ~1669 cm^{-1}, must result from the stretching vibrations of the formyl substituents [9]. This additionally confirms the assignment of the porphyrin groups of this oxidase to type-a hemes [6]. Such hemes also contain a vinyl substituent, which gives rise to a RR-active stretching vibration typically located at ~1620–1625 cm^{-1}. In fact, the poorly resolved vibrational structure in this region reveals a band at ~1623 cm^{-1}. There are apparently two further bands at 1640 and 1613 cm^{-1} which are attributed to the ν_{10} modes of heme a (6cLS) and a_3 (6cHS), respectively. Again, we note a good agreement with the data reported for beef heart cytochrome oxidase.

Upon complete reduction of aa_3, significant changes are observed in the RR spectrum. The most pronounced effect is observed for the ν_4 mode, which shifts down to 1358 cm^{-1} (Fig. 3A,B). This mode is a nearly pure C–N stretching vibration. Its frequency lowering in ferrous

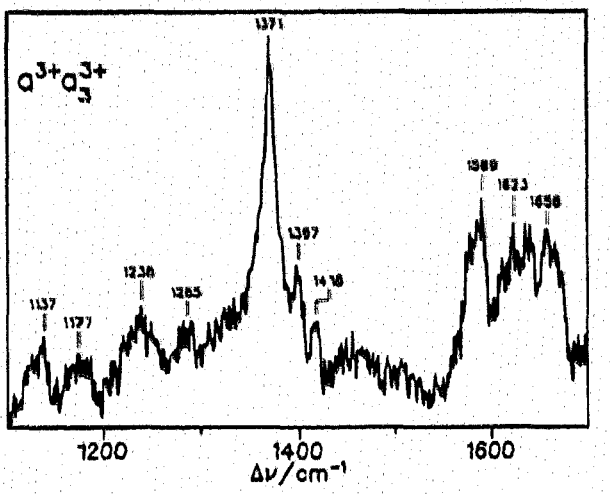

Fig. 1. RR spectrum of the fully oxidized cytochrome aa_3 ($a^{3+}a_3^{3+}$) excited at 413 nm.

hemes originates from the increased back donation of electron density from the d_π orbitals of the iron to the antibonding MOs of the porphyrin, which substantially weakens the strength of the C–N bonds [11,12]. An additional effect of reduction is the increase of the effective radius of the iron compared to the ferric ion. According to the core-size/frequency relationships derived from model compounds, this should lead to a frequency lowering of the spin state marker bands [11,12]. This is in fact observed for ν_2 of a_3^{2+} which shifts down by 6 cm^{-1} to 1569 cm^{-1} (Fig. 2C). Also the intensity increase at ~1625 cm^{-1} at the expense of the 1640 cm^{-1} band can be rationalized in terms of a downshift of the ν_{10} mode.

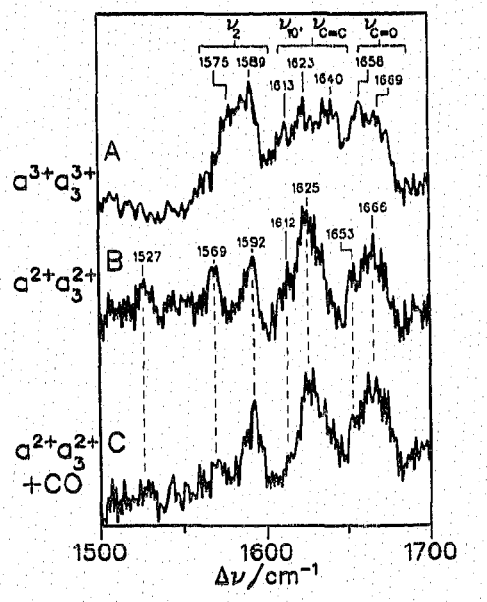

Fig. 2. RR spectra of cytochrome aa_3 in the marker band region excited at 413 nm. (A) Fully oxidized cytochrome aa_3 ($a^{2+}a_3^{2+}$). (B) Fully reduced cytochrome aa_3 ($a^{2+}a_3^{2+}$). (C) Carbon monoxide complex of the reduced cytochrome aa_3 ($a^{2+}a_3^{2+}$ + CO).

On the other hand, the ν_2 mode of a^{2+} is even at a higher frequency (1592 cm^{-1}) compared to a^{3+}. Deviation from the expected value has frequently been observed in ferrous 6cLS iron porphyrins and were attributed to back-donation effects [11,12]. Also the ν_{10} mode of heme a_3 remains at about the same frequency in both oxidation states. Spiro and coworkers pointed out that at such a large expansion of the tetrapyrrole macrocycle which is required to accommodate a 6cHS ferrous ion, at least for some of the porphyrin modes the linear core-size/frequency relationships fail [12].

The region of the C=O stretching vibration exhibits a broad peak, similar to the oxidized form, although individual components cannot be identified.

In a previous study it was shown that the reduced heme a_3 is capable of binding carbon monoxide as reflected by a shift of the Soret band maximum above 440 nm [5]. Figs. 2C and 3C show the RR spectra of this CO-complex. In the ν_4 band region we note two bands at 1362 and 1372 cm^{-1} (Fig. 3C). The latter band is attributed to the CO-bound a_3^{2+}. The considerably higher frequency compared to the unbound a_3^{2+} implies that the coordination of CO to the heme iron compensates the effect of back donation so that ν_4 shifts up to a value close to that of the oxidized species. The CO-ligand possesses empty π^* orbitals which can effectively compete with the antibonding MOs of the porphyrin for electron density. Similar frequency upshifts, although not so pronounced, have frequently been observed in CO-binding hemes [15,16].

The 1362 cm^{-1} band must originate from the ν_4 mode of a^{2+}. The RR spectrum of this heme should be largely

Table 1

Comparison of the high-frequency modes (cm^{-1}) of heme *a* and a_3 in cytochrome aa_3 of *S. acidocaldarius* and eucaryotic cytochrome oxidase

State	Species	Modes				
		ν_4	ν_2	ν_{10}	$\nu C=C$	$\nu C=O$
a^{3+}	*Sulfolobus*	1371	1589	1640	1623	1658
	beef[a]	1371	1590	1641	1622[c]	1650
	wheat[b]	1371	1586	1638	-[d]	1657
a_3^{3+}	*Sulfolobus*	1371	1575	1613	1623	1669
	beef[a]	1373	1572	1615	1622	1676
	wheat[b]	1371	1576	-[d]	-[d]	1677
a^{2+}	*Sulfolobus*	1362	1592	1625[c]	1625[c]	1653
	beef[a]	1354	1585	1614	1623	1610
	wheat[b]	1356	1587	1618	1627	1610
a_3^{2+}	*Sulfolobus*	<1358	1569	1612	1625[c]	1666
	beef[a]	1356	1579	1607	1623	1665
	wheat[b]	1361	-[d]	-[d]	1627	1665

[a]Beef heart cytochrome oxidase, adopted from [13]
[b]Wheat germ cytochrome oxidase, adopted from [13]
[c]Adopted from [14]
[d]Not reported
[e]Not resolved into the $\nu C=C$ and ν_{10} modes

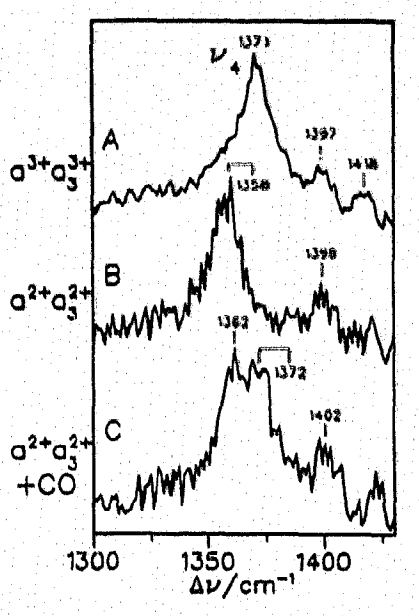

Fig. 3. RR spectra of cytochrome aa_3 in the ν_4-band region excited at 413 nm. (A) Fully oxidized cytochrome aa_3 ($a^{3+}a_3^{3+}$). (B) Fully reduced cytochrome aa_3 ($a^{2+}a_3^{2+}$). (C) Carbon monoxide complex of the reduced cytochrome aa_3 ($a^{2+}a_3^{2+}$ + CO).

unaffected by CO-binding to a_3^{2+}. Since there is a frequency shift compared to the common envelope of the ν_4 modes in $a^{2+}a_3^{2+}$ (Fig. 3B), this implies the corresponding band of a_3^{2+} to be located slightly below 1358 cm^{-1} so that its overlap with 1362 cm^{-1} of a^{2+} leads to the peak maximum at 1358 cm^{-1}.

In the spin state marker band region, the bands attributable to a_3^{2+} disappear due to the formation of the carbon monoxide complex as indicated by the loss of RR intensity at 1569, 1527, and 1612 cm^{-1} (Fig. 2B,C). They are expected to shift to higher frequencies [15,16] and may accidentally coincide with those of a^{2+}. Their contribution to the RR spectrum, however, may be lower than for the modes of a^{2+} due to the red-shift and the decrease of the oscillator strength of the Soret band [5].

4. DISCUSSION

The RR spectra of cytochrome aa_3 demonstrate that heme *a* and a_3 are in the 6cLS- and 6cHS-configuration, respectively, confirming previous conclusions drawn from other spectroscopic studies [5]. For the fully oxidized form, the comparison with the RR data obtained from eucaryotic cytochrome oxidase (Table I) reveals far-reaching similarities suggesting that the kind of axial ligands of the heme irons is the same in the various species.

Spectral differences larger than 3 cm^{-1} are only noted for ν_4 and ν_2 of a^{2+} and ν_2 of a_3^{2+} (Table I). The considerably higher frequency of ν_4 in a^{2+} compared to beef heart cytochrome oxidase may be taken as an in-

dication for weaker electron density delocalization into the π^* orbitals of the porphyrin [11,12,17], for example, due to different hydrogen bonding interactions of the axial ligands. A similar explanation may hold for the upshift of ν_2 of this heme. On the other hand, the ν_2 frequency of a_3^{3+} of beef heart is unusually high (1579 cm^{-1}) while in *S. acidocaldarius* it is close to the frequency observed for a protein-free 6cHS heme *a* (1567 cm^{-1}; [18]).

The formyl stretching vibrations can give insight into the structure of the heme pockets since their frequencies are sensitive to hydrogen bonding interactions [9,19]. In the fully oxidized state of *S. acidocaldarius*, these bands are at 1658 cm^{-1} (heme a^{3+}) and 1669 cm^{-1} (heme a_3^{3+}) pointing to weakly hydrogen-bonded and a non-hydrogen-bonded carbonyl group, respectively. These frequencies are about in the same range observed for eucaryotic cytochrome oxidase (Table I). Reduction of the heme iron causes a downshift of this mode. For a 6cHS heme a_3^{2+} in an aprotic environment, it is generally found at -1665 cm^{-1} [9,19], which is in good agreement with the observed peak maximum in the RR spectrum of $a^{2+}a_3^{2+}$ (Fig. 2B).

A striking difference with respect to eucaryotic enzymes is observed for the C=O stretching of heme a^{2+}. In beef heart cytochrome oxidase, this mode is as low as 1610 cm^{-1} reflecting a considerable increase of the hydrogen bond strength compared to the oxidized form [9,19]. Although the RR spectrum of $a^{2+}a_3^{2+}$ presented in this work also shows a shoulder at 1612 cm^{-1} (Fig. 2B), such an assignment is unlikely in the case of *S. acidocaldarius*. In the CO-complex (Fig. 2C), the RR intensity at 1612 cm^{-1} has clearly decreased suggesting the assignment to a mode of heme a_3^{2+} (i.e. ν_{10}). Moreover, the broad peak at 1666 cm^{-1} in the RR spectrum of $a^{2+}a_3^{2+}$ (Fig. 2B) which exhibits a half-width of approximately 25 cm^{-1} is a strong indication for more than one band contributing to the RR signal. Consequently, we conclude that the C=O stretching of a_3^{2+} is also located in this region. It may be that it corresponds to the shoulder at ~ 1653 cm^{-1}; however, an unambiguous detection is not possible due to the poor S/N ratio. Independent of the exact position of this band, such a high frequency would imply that the formyl group of a^{2+} is free of any hydrogen bonding interactions which is in sharp contrast to beef heart cytochrome oxidase [9,19].

The optical absorption spectra are in a qualitative agreement with this conclusion inasmuch as the blue shift of the Soret- and α-bands in the reduced state indicate a significantly weaker hydrogen bond strength compared to beef heart cytochrome oxidase [5,19]. However, these data are not compatible with an aprotic milieu around the formyl group. Thus, it is likely that there is an additional parameter which causes such an unusually high frequency of the C=O stretching vibration. Since also the porphyrin modes ν_4 and ν_2 of heme a^{2+} exhibit substantial upshifts it is tempting to ascribe both effects to a common origin, i.e. modifications of the electron density distribution as discussed above.

In mammalian cytochrome oxidase the redox linked change of hydrogen bonding interactions was interpreted by Babcock and Callahan [19] in terms of an involvement of the heme *a* formyl group in proton pumping, assuming that reduction of heme *a* may initiate a switch by conformational constraint. According to the above data this mechanism would appear unlikely for the *Sulfolobus* enzyme. However, so far it remains to be shown that this single subunit oxidase is capable of proton pumping, because the functional complex may comprise more than one polypeptide subunit in the intact membrane. On the other hand, the use of quinones instead of cytochrome *c* as a substrate is a specific property of the interaction domains of the protein; eventually, since a typical copper A is absent, a tightly bound caldariella quinone [5] may replace its role in electron transport.

Acknowledgement: We like to thank Prof. K. Schaffner for continuous encouragement and support.

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