Resonance Raman spectroscopy and enhanced photoreducibility for the 420 nm pulsed form of cytochrome oxidase

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Resonance Raman (RR) spectra, with 413.1 nm Kr^+ laser excitation, are reported for cytochrome oxidase in resting, reduced, and 428 nm (oxygenated) forms, and for the first time, in the 420 nm (pulsed) forms [(1984) J. Biol. Chem. 259, 2073–2076]. The differences between the resting, 420 nm, and 428 nm forms' RR spectra are small. All these forms contain Fe^{III} only, as indicated by single ν_4 bands at $\sim$1371 cm^{-1}, and the reoxidized forms show partial conversion from high- to intermediate- or low-spin heme a_3 (intensity shift from 1575 to 1588 cm^{-1} for ν_2). The 420 nm form differs strikingly from both the 428 nm and resting forms, however, in being much more readily photoreduced by the laser illumination. This property is linked to the protein conformational change believed to be responsible for the greater accessibility to exogenous ligands of the heme a_3 in the 420 nm form.

Cytochrome oxidase *Pulsed oxidase* *Oxygenated oxidase* *Resonance Raman spectroscopy*

1. INTRODUCTION

Cytochrome oxidase (ferrocytochrome c:O_2 oxidoreductase, EC 1.9.3.1) is the terminal enzyme in the respiratory electron transfer chain, catalyzing the 4-electron reduction of dioxygen to water [1]. The resting enzyme (as isolated) is characterized by an absorption spectrum with a Soret maximum at 418–420 nm and weak absorption in the α-region [2]. Reduction with excess dithionite, followed by reoxidation of the enzyme by oxygen results in a shift of the Soret maximum to 428 nm and enhanced α-band absorption [3]. This 'oxygenated' form [based on earlier results [9,10], we equate the 420 nm form with 'pulsed' oxidase and the 428 nm form with 'oxygenated' (pulsed-peroxidase) oxidase] of cytochrome oxidase exhibits increased reactivity towards enzyme substrate and exogenous ligands relative to the resting form. Thus this form has been the subject of numerous studies [3–8], although its physiological relevance is still a matter of some debate. It was recently shown that reoxygenation of anaerobically reduced oxidase in the strict absence of peroxide yields a protein form (the 420 nm form) with an electronic spectrum very similar to the resting form, but with significantly increased reactivity [9,10]. This 420 nm form is converted to the traditional 428 nm form upon addition of peroxid [9,10].

Here we report the first resonance Raman (RR) characterization of the 420 nm pulsed form of cytochrome oxidase. Using Soret excitation we compare the RR spectra of the resting, reduced, 420 nm, and 428 nm forms of the enzyme, concentrating our efforts on the spectral region between 1300 and 1700 cm^{-1} where heme oxidation and spin state marker bands are known to occur [11]. By study of the laser power dependence of the RR

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spectra of these forms we have determined that the 420 nm form undergoes much more efficient photoreduction than either the resting or 428 nm forms. We interpret this result as indicating a protein conformational change between the resting and 420 nm cytochrome oxidase as previously suggested [9,10].

2. EXPERIMENTAL

Bovine cytochrome oxidase was prepared by the method of Volpe and Caughey [12] as modified in our laboratories (to be reported). To minimize sample fluorescence for Raman studies, the detergent used to isolate the enzyme (Tween 20) was exchanged for lauryl maltoside by dialysis against 50 mM phosphate buffer (pH 7.4), 0.5% lauryl maltoside, followed by dilution, and reconcentration. Final oxidase concentration was 60 μM (using an extinction coefficient of 79.6 $mM^{-1} \cdot cm^{-1}$ at 418 nm for the resting form) in 50 mM phosphate buffer (pH 7.4), 0.5% lauryl maltoside. Solutions of resting enzyme which were used to produce the 420 nm species additionally contained 50 nM catalase as a peroxide scavenger. Catalase was purchased from Sigma, all other reagents were the highest grades commercially available and used as received. The 420 nm and 428 nm forms were prepared by reduction of the resting enzyme (with and without catalase, respectively) with a minimum volume of saturated sodium dithionite solution (under an N_2 atmosphere for 20 min) followed by reoxygenation by vigorous mixing with air in a vortex mixer for 1 min. In the case of the 420 nm form, after Raman data acquisition (5–10 min) about 10 mM H_2O_2 was added to produce the peroxide adduct of this form. This addition caused excessive bubbling due to the reaction of peroxide with catalase, and for this reason a very small amount of antifoam (Thomas Scientific) was added. Nonetheless, some bubbling was still present which lowered the signal-to-noise ratio obtained for the RR spectrum of this form (fig.1d). All species were characterized before and after acquisition of Raman spectra by absorption spectroscopy using a Hewlett-Packard 8450A diode array UV-VIS spectrophotometer. The actual Soret maxima observed for these samples were: resting, 416–418 nm; reduced, 442 nm; pulsed, 421–422 nm; pulsed-peroxide, 427 nm; oxygenated, 426 nm. Raman spectra were obtained in resonance with the Soret absorption band of the oxidase forms using 413.1 nm excitation from a c.w. Kr^+ laser, and a 90° scattering geometry from a capillary tube. To avoid photoreduction the samples were contained in a glass reservoir (volume 2 ml) which was cooled to 4°C, in an ice bath, and continually flowed through the capillary, at a constant rate, by use of a peristaltic pump (Masterflex). The scattered light was focused onto the entrance slit of a Spex 1870 single monochromator equipped with a PAR OMA-II R eticon detector. Spectra were accumulated for 10 min unless otherwise noted.

3. RESULTS AND DISCUSSION

Fig.1 shows the RR spectra of the various cytochrome oxidases forms studied here between 1340 and 1700 cm^{-1}. Soret excited RR spectra of the 428 nm [13,14] reduced, and resting [15] forms of the enzyme have been previously reported, and our data are in qualitative agreement with these earlier results. The intense oxidation state marker band, ν_4, occurs at 1358 cm^{-1} for the reduced enzyme as expected for an Fe^{II} oxidation state for both hemes [11], and at ~1371 cm^{-1} for resting and reoxidized forms. This result implies that the heme irons remain in the Fe^{III} state in both the 420 and 428 nm forms of the enzyme; had the heme a_3 iron been oxidized to Fe^{IV}, a ν_4 band should have been seen at a frequency near 1380 cm^{-1}, as in horseradish peroxidase compound II [16]. To check the possibility that initially formed Fe^{IV} is converted to Fe^{III} by a rapid laser-induced reaction we monitored ν_4 during signal acquisition with the OMA; within 20 s of laser illumination (with laser power as low as 5 mW) of the recirculating sample, the band positions were ~1371 cm^{-1}.

In the 1400–1600 cm^{-1} region small but reproducible differences are seen among the various forms of the enzyme. Of principal interest is the shift in intensity, between the resting and reoxidized forms, from the band at 1575 cm^{-1} to that at 1588 cm^{-1}. These are assigned [15] to the ν_2 skeletal modes of high- and low-spin hemes, respectively, and the shift has been interpreted [13,14] as evidence for conversion of heme a_3 from high-spin in the resting enzyme to low- or intermediate-spin in the 428 nm form. The rem-

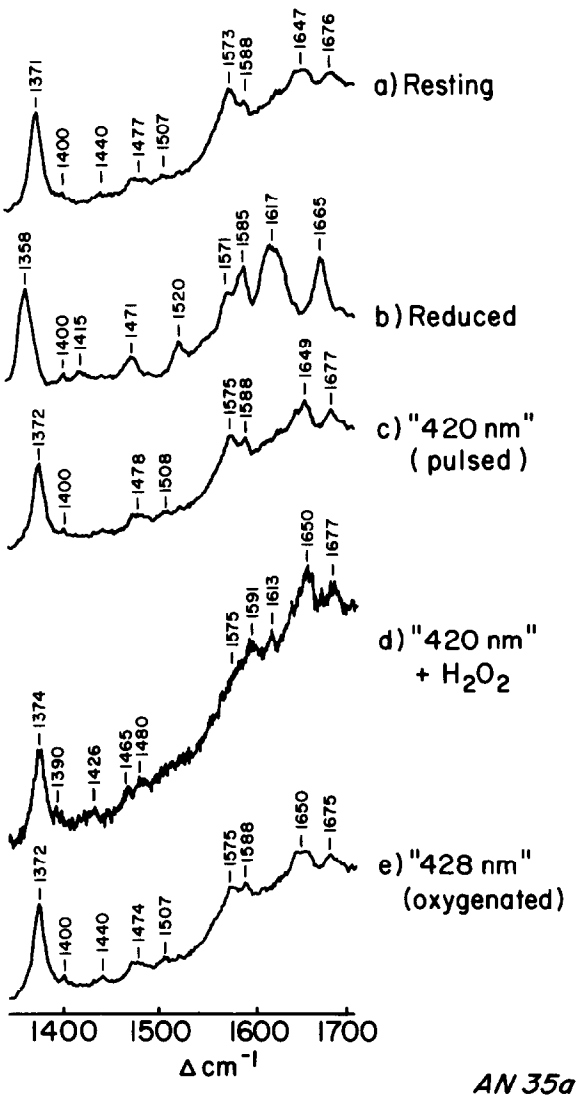

Fig.1. Soret excited RR spectra of bovine cytochrome oxidase in the resting (a), reduced (b), 420 nm (c), 420 nm plus H_2O_2 (d) and 428 nm form (e). All spectra were obtained with 50 mW laser power (at the source) of the 413.1 nm line from a Kr^+ laser. See section 2 for further details.

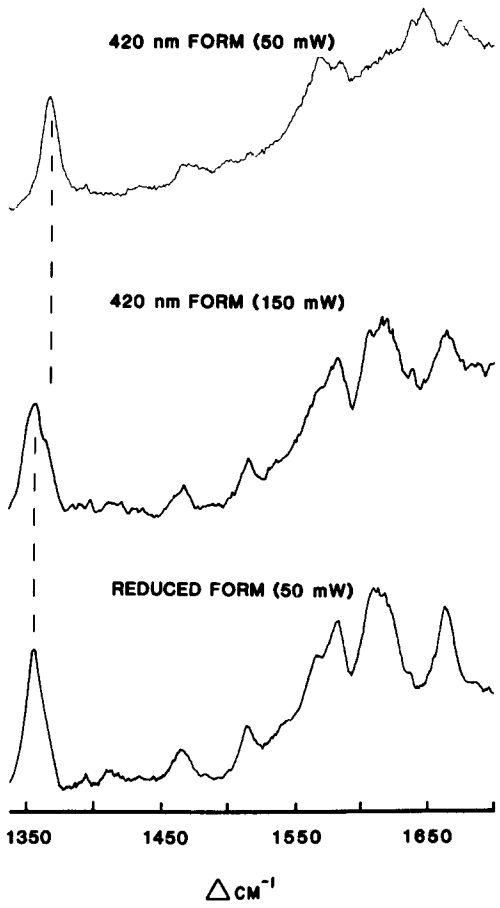

Fig.2. Laser power dependence of the Soret excited RR spectrum of the 420 nm pulsed form of cytochrome oxidase. The top spectrum was taken with 150 mW laser power (at the source), the middle spectrum was with 50 mW laser power, and the bottom spectrum is that of the dithionite-reduced enzyme taken with 50 mW of laser power. Each spectrum was accumulated for 5 min. See section 2 for further details.

nant intensity at 1575 cm^{-1} may be due to partial decay during the spectral acquisition [13] and/or incomplete peroxide ligation in the oxygenated form [8]. However, the possibility of a mixed-spin heme a_3 within a homogeneous protein cannot be excluded. In this spectral region the 420 nm form shows a distinct band at ~1575 cm^{-1} which almost disappears upon H_2O_2 addition. This may imply a larger population of high-spin heme a_3 in the 420 nm form relative to the peroxide adduct.

Wrigglesworth [17] has found that the 605 nm absorption band, attributed to the 420 nm form [10] can also be induced by long incubations of resting cytochrome oxidase with H_2O_2. This brings up a question as to whether heme a_3 of the 420 nm form is 6-coordinate, or 5-coordinate as suggested by Kumar et al. [10] on the basis of their work and that of Beinert et al. [18]. Our results are not inconsistent with either of these two possibilities. Table 1 summarizes these results and offers ten-

Table 1

Band frequencies (in cm^{-1}) and tentative assignments for Raman modes of cytochrome oxidase

Resting	Reduced	420 nm	420 nm + H_2O_2	428 nm	Assignment[a]
1676	1665	1677	1677	1675	ν_{CO}
1647	–	1649	1650	1650	$\nu_{CO\ .H}(a)$
–	1617[b]	–	–	–	$\nu_{10}(a) + \nu_{C=C}$
–	–	–	1613	–	?
1588	–	1588	1591	1588	ν_2(low spin)
–	1585	–	–	–	$\nu_{37X}(a)$
1573	–	1575	1575	1575	ν_2(high spin)
–	1571	–	–	–	$\nu_{38X}(a)$
–	1520	–	–	–	$\nu_{11}(a_3)$
1507	–	1508	–	1507	$\nu_3(a)$
1477	–	1478	1480	1474	$\nu_3(a_3)$
–	1471	–	1465	–	$\nu_{28}(a)$
1440	–	–	1426	1440	$\nu_{28}(a_3)$
–	1415	–	–	–	?
1400	1400	1400	1390	1400	ν_{28}/ν_{20}
1371	1358	1372	1374	1372	ν_4

[a] Assignments taken from [15]. Bands which are unique to either cytochrome *a* or a_3 are indicated in parentheses

[b] This band is composed of two bands at 1611 and 1624 cm^{-1} which are not resolved in our spectra

–, band not observed

tative assignments for the observed Raman bands.

Despite the spectral similarities, the 420 nm form turns out to be much more readily photoreduced than the resting or 428 nm forms, as demonstrated in fig.2. With 150 mW of 413.1 nm laser power (at the source) the 420 nm form is mostly reduced, as evidenced by the 1358 cm^{-1} ν_4 band, within the 5 min spectral acquisition time, despite being flowed continuously through the sample capillary. Under these conditions neither the 428 nm nor the resting form shows evidence of reduction, although the resting form is known [19,20] to be photoreduced by continuous laser illumination of a stationary sample. These observations are reminiscent of the results reported by Kitagawa and Nagai [21] on aquo-methemoglobin, which shows autoreduction upon laser illumination, but only after addition of inositol hexaphosphate, which switches the protein quaternary structure from R to T. Also the photoreducibility of the giant hemoglobin of *T. japonica* has been shown to be dependent on the subunit assembly of the protein [22]. While the endogenous electron donor in these samples has not been identified, it appears that high-spin Fe^{III} heme is generally susceptible to photoreduction, at a rate that depends on protein conformation. Kumar et al. [9,10] have suggested that the differences between the resting and 420 nm oxidase forms are due to a protein conformational change that opens the heme a_3 pocket, making it more accessible to exogenous ligands. The greatly enhanced photoreducibility may be another manifestation of this conformational difference.

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