Diheme cytochrome *c*-554 from *Nitrosomonas*

Soret resonance Raman indication of an unusual ferric 5-coordinate structure

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The diheme cytochrome *c*-554 which participates in ammonia oxidation in the chemoautotroph, *Nitrosomonas europaea* has been studied by Soret excitation resonance Raman spectroscopy. The Raman spectrum of reduced cytochrome *c*-554 at neutral pH is similar classical 6-coordinate low-spin ferrous mammalian cytochrome *c*. In contrast, the spectrum of ferric cytochrome *c*-554 suggests a 5-coordinate state which is unusual for *c* hemes. The oxidized spectrum closely resembles that of horseradish peroxidase (HRP) or cytochrome *c* peroxidase (CcP) at pH 6.4. The narrow linewidth of the heme core-size vibrations indicates that both heme irons of *c*-554 have similar geometries.

Nitrosomonas *Cytochrome c-554* *Raman spectroscopy* *Nitrification* *5-Coordinate heme* *c'-like cytochrome*

1. INTRODUCTION

The chemoautotroph *Nitrosomonas europaea* derives energy for growth by oxidizing ammonia to nitrite. Oxidation of ammonia is believed to involve hydroxylamine (NH_2OH) or a closely related compound as an intermediate which is oxidized by the enzyme hydroxylamine oxidoreductase (HAO). Electrons are thought to pass successively from NH_2OH to HAO to cytochrome *c*-554 to cytochrome *c*-552 and then to a terminal oxidase [1]. Recent studies suggest that cyt *c*-554, a single subunit, 25 kDa protein with two *c* type hemes [1], is involved in the cell-free oxidation of ammonia [2].

Characterization of cytochrome *c*-554 by a variety of spectroscopic methods has not given a clear indication of the spin state of the oxidized hemes at neutral pH. Cytochrome *c*-554 has an unusual EPR signal at pH 7.0 and 10 K; a major broad resonance around $g = 4$ and an unresolved $g = 2$ component are observed [3,4]. The data suggest the existence of unusual spin interactions. Although the overall shape of the signal observed for cytochromes *c*-554 and *c'* is similar, the $g = 4$ resonance in *c*-554 is significantly lower than a corresponding resonance in *c'* [5,6]. Under the conditions of measurement and using only the EPR data, a mixture of low-spin and high-spin forms can be ruled out but the assignment of an intermediate ($S = 3/2$) spin state cannot be made with certainty. The room temperature ^{1}H-NMR spectrum (270 MHz) of cytochrome *c*-554 has 4 resonances between 100–600 ppm typical of high-spin contact shifted heme methyl resonances and more than 4 resonances below 35 ppm which may be contact shifted low-spin heme methyl resonances [4]. By NMR spectroscopy there is no clear indication of intermediate spin. These results and their apparent conflict with the EPR data sug-

Abbreviations: HRP, horseradish peroxidase; HAO, hydroxylamine oxidoreductase; CcP, cytochrome *c* peroxidase

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gest that the different conditions of measurement for the two magnetic techniques may have introduced shifts in the physical state of the hemes. Soret excitation resonance Raman spectroscopy has the potential to resolve the paradoxical magnetic resonance results in that this method is applicable to samples in the liquid phase at room temperature and is sensitive to heme stereochemistry [7]. In the experiments reported here, we have used this technique to characterize oxidized and reduced *c*-554 at neutral pH.

2. MATERIALS AND METHODS

Cells of *N. europaea* were grown in batch cultures and collected in logarithmic phase of growth, as described before [8]. Cytochrome *c*-554 was prepared according to [1] and stored in 0.6 M ammonium phosphate solution (pH 7) at −20°C until use. The cytochrome was dissolved in 50 mM potassium phosphate solution (pH 7.0) for the Raman experiments. Reduction of cyt *c*-554 was by the addition of a few crystals of solid dithionite. Raman spectroscopy (excitation at 406.7 nm) was carried out at 4°C as in [9]. Optical spectra were recorded before and after the Raman spectra to monitor sample integrity.

3. RESULTS

The resonance Raman spectra of reduced and oxidized cytochrome *c*-554 at neutral pH are shown in fig.1A and B, respectively. The absorbancy of samples in the Soret region was 1. The prominent vibrations observed for oxidized and reduced cyt *c*-554 in the high frequency region are collected in table 1 and assigned, as described below, according to the normal coordinate analysis in [10] and by analogy to the observations in [7,11]. As judged by optical absorption spectra, laser irradiation of the samples did not damage the protein.

4. DISCUSSION

The optical spectrum of reduced *c*-554 has characteristics typical of a low-spin, ferrous, *c*-type heme with well-resolved and intense alpha and beta bands [1]. In accord with its optical properties, the Raman spectrum of the reduced protein

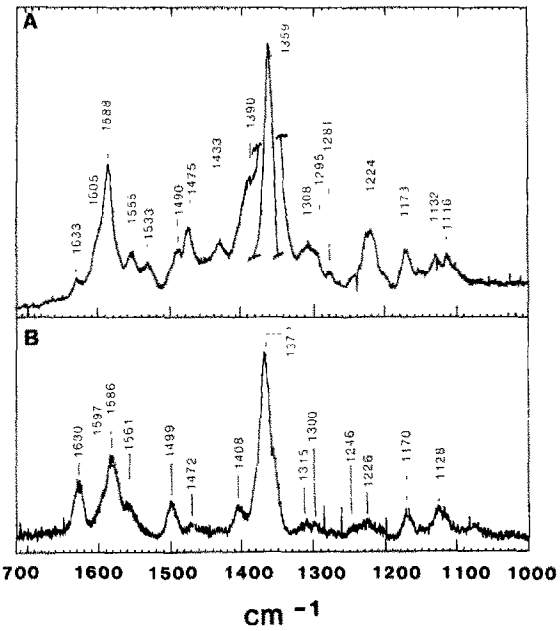

Fig.1. Soret excitation resonance Raman spectra of reduced and oxidized cytochrome *c*-554 from *N. europaea* (λ_{ex} = 406.7 nm, pH 7.0). (A) Raman spectrum of dithionite reduced cytochrome *c*-554 at pH 7 with excitation at 406.7 nm. The laser power at the sample was 20 mW. A scan rate of 50 cm^{-1}/min and a time constant of 1 s were used in recording the spectrum. (B) Raman spectrum of oxidized cytochrome *c*-554 at pH 7. The *y*-axis sensitivity is three times greater than in A; other conditions were as in A.

(fig.1A) resembles that of mammalian ferrocytochrome *c* [12]. This is apparent in the bands in the 1100–1300 cm^{-1} region, which are essentially identical in both frequency and intensity (relative to the strong 1359 cm^{-1} ν_4 mode) to those observed in the mammalian protein with excitation at 413.1 nm [12], and in the frequencies of ν_{37}, ν_2, ν_{38}, ν_{11} and ν_3, all of which are typical of 6-coordinate, low-spin ferrous hemes [11]. Small differences between the frequencies for these modes in ferrous *c*-554 and cytochrome *c* [12,13] may reflect the 4 nm shift in the α-band maximum of *c*-554 or the presence of a very weak 430 nm shoulder [1] not found in the mitochondrial cytochrome *c*. The unusually high frequency of ν_{28} may also reflect structural features of the heme pocket specific to the cyt *c*-554 binding site. Mode ν_{10}, which occurs at 1622 cm^{-1} in reduced mitochondrial cytochrome *c* [12], has not been

Table 1

Mode assignments for cytochrome *c*-554 and related species

Mode symmetry[d]	Cyt c^{2+}-554[a]	Cyt c^{3+}-554[a]	Fe^{3+} etio-porphyrin Cl^{-}[b]	HRP[b]	CcP	
					pH 6.4[c]	pH 7.6[c]
ν_{10} (B_{1g})	[e]	1630	1632	1630	1632	1643
ν_{37} (E_u)	1605	1597	–	–	–	–
ν_2 (A_{1g})	1588	1586	1585	1573	1574	1581
ν_{38} (E_u)	1555	–	–	–	–	–
ν_{11} (B_{1g})	1533	1561	–	–	1552	–
ν_3 (A_{1g})	1490	1499	1496	1498	1496	1508
ν_{28} (B_{2g})	1475	1472	–	–	–	–
ν_{29} (A_{1g})	1433	1408	–	1430	–	–
ν_{29} (B_{2g})	1390	–	–	–	–	–
ν_4 (A_{1g})	1359	1372	1373	1375	1375	1377
ν_{21} (A_{2g})	1308	1315	–	–	1314	1316

[a] Here
[b] From [7]
[c] From [18]
[d] From [10]
[e] Not assigned, see text

assigned in table 1 as the normally weak intensity of this mode with Soret excitation and possible incomplete reduction of the protein may have obscured this vibration. The appearance of ν_{21} at 1308 cm^{-1} and the absence of modes between it and ν_4 at 1359 cm^{-1} confirm the *c* type heme nature of *c*-554. In general, the Raman spectrum of reduced cyt *c*-554 at neutral pH indicates that a large majority of the heme iron is 6-coordinate and low-spin. This conclusion is in agreement with preliminary Mossbauer data for ^{57}Fe enriched ferrous cytochrome *c*-554 at 4 K (unpublished) which shows that the majority of the iron is in the low-spin state. Reduced cytochrome *c*-554 is clearly different from ferrous cytochrome *c'* which is high-spin [14].

A different conclusion is reached as to the coordination geometries of the hemes in oxidized cytochrome *c*-554. In ferric hemes and heme proteins, three porphyrin skeletal modes, ν_{10}, ν_2 and ν_3, which are sensitive to the ligation and spin state of the heme iron and the nature of substituents at the periphery of the porphyrin macrocycle [7,15,16] are prominently enhanced by Soret excitation. Comparison of the spectrum of oxidized cyt *c*-554 to those of the models in [7] shows that ν_{10} and ν_3 in *c*-554 are similar in frequency to the corresponding vibrations in both oxidized horseradish peroxidase (HRP) and ferric etioporphyrin chloride (see table 1). Furthermore, the frequency of ν_2 in cytochrome *c*-554 corresponds closely to ν_2 in the ferric etioporphyrin chloride model, but is 13 cm^{-1} higher in frequency than ν_2 in HRP. The latter observation is rationalized by the fact that ν_2 shows a strong dependence on peripheral substituents on the heme [9,10]; *c*-type hemes are similar to iron etioporphyrins in that all β-carbon substituents are saturated whereas the 2,4-divinyl substitution in the protoheme of HRP alters the ν_2 frequency independent of the iron coordination geometry. From the frequencies of these three prominent modes, a 5-coordinate geometry in which the iron is out of the heme plane is suggested for oxidized cyt *c*-554. The other modes in the high frequency region have been assigned in analogy with the examples in [11] and are also consistent with a 5-coordinate geometry. Raman data on cytochrome *c* peroxidase (CcP) [17,18] show a similar coordination geometry for the heme iron at pH 6.4 but not at pH 7.6, where a low-spin, 6-coordinate geometry is inferred. These data have been included in table 1 as well.

The determination of the spin states of the irons in oxidized *c*-554 is less clear cut. A low-spin (i.e., $S = 1/2$) state is unlikely as this usually requires a 6-coordinate in-plane iron ligation geometry, which is clearly not indicated by the Raman data. Moreover, the linewidths of the observed vibrations show no evidence of broadening when compared to those of simpler heme model compounds [7,15,16]. This precludes the occurrence of a substantial thermal spin-state equilibrium in the sample, at least at room temperature, and indicates that the two hemes of *c*-554 are in similar environments. It also suggests that the resonances observed in ^{1}H-NMR spectroscopy (i.e., in the presence of a strong magnetic field) in the region below 35 ppm [3,4] are either contact shifted low-spin methyl resonances from a very small fraction of the molecules in solution at neutral pH or have their origin in some other phenomenon. However, the Raman data do not allow one to decide between a high-spin ($S = 5/2$), intermediate spin ($S = 3/2$) or a quantum-mechanically mixed-spin [($S = 5/2, 3/2$) or ($S = 1/2, 3/2$)] state in the oxidized protein. This uncertainty arises because Raman is not sensitive to paramagnetism per se, but rather to the structure about the iron center. Nonetheless, the Raman parameters indicate a coordination geometry for cyt *c*-554 similar to that of ferric HRP and CcP at pH 6.4. The magnetic properties which result from such a structure appear to be somewhat ambiguous in that HRP could, in fact, be in a mixed-spin state [6], whereas CcP at pH 6.4 has been assigned as high-spin [18,19]. That *c*-554 appears to have an open coordination position at the irons of the oxidized enzyme, however, raises interesting possibilities as to its biological function in light of the peroxidase activities of HRP and CcP.

In conclusion, the Raman data suggest that both heme irons of oxidized cyt *c*-554 at neutral pH have a 5-coordinate out-of-plane conformation, unusual for *c*-hemes. Neither of the irons is in a 6-coordinate low-spin state, but the actual spin state cannot unequivocally be assigned. Upon reduction, the coordination geometry shifts; an additional ligand(s) apparently binds to each of the irons to produce a 6-coordinate, low-spin ferrous species.

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