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RESONANCE RAMAN STUDIES OF A *c* TYPE ALGAL CYTOCHROME

DEUTERIUM SHIFTS AND A COMPARISON WITH MAMMALIAN CYTOCHROME *c*

TOMOKO YAMAMOTO, GRAHAM PALMER and HENRY CRESPI

Biophysics Research Division, University of Michigan, Ann Arbor, Michigan, Department of Biochemistry, Rice University, Houston, Texas, and Argonne National Laboratories, Argonne, Ill. (U.S.A.)

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SUMMARY

A *c* type cytochrome isolated from *Synechococcus lividus* grown on water and $^2\text{H}_2\text{O}$ media, has been studied by resonance Raman spectroscopy. The spectra were taken on the oxidized and reduced protein with excitation within the Soret band at 441.6 nm to determine whether individual resonance Raman bands of the heme shift upon deuterium substitution and also to provide a comparison with the spectra of horse heart cytochrome *c*.

Some of the shifts observed with the deuterated heme *c* are larger than the corresponding shifts in *meso*-deuterated metalloporphyrins suggesting mixing of peripheral substituent vibrations with the skeletal modes of the porphyrin macrocycle.

The algal cytochrome exhibits resonance Raman spectra roughly similar to those of horse heart cytochrome *c*, consistent with its optical absorption spectra which is typical of *c* type cytochromes, although a detailed comparison reveals noteworthy differences between the spectra of the two proteins; this may be a reflection of the effect of non-methionine ligands and protein environment on the vibrations of the *c* type heme in the algal cytochrome.

INTRODUCTION

Resonance Raman spectra have been reported for a number of hemoproteins such as cytochrome *c*, hemoglobin, etc. [1]. One of the unanswered questions is the extent to which peripheral substituents contribute to the resonance Raman spectra of these compounds. These contributions may originate for two reasons: (1) the substituent vibrations are mixed in with the skeletal vibrations and (2) the substituent vibrations themselves appear resonance enhanced. Since all the peripheral substituents, methyl, vinyl, propionic acid groups, etc. contain hydrogen, complete deuterium substitution offers a possibility of distinguishing among bands depending on the magnitude of the deuterium shifts. As there are four *meso* hydrogens on the porphyrin ring itself, studies with partially deuterated hemes would also be helpful

particularly if the deuterium shifts resulting from *meso* deuterium substitution can be distinguished from those attributable to the peripheral substituents.

A second important but unsettled question is whether or not the resonance Raman spectra of the heme reflect the effect of the ligands and protein environment. Loehr and Loehr [2] concluded from their studies of cytochrome *c* and microperoxidase that the resonance Raman spectra do not reflect changes in the protein environment around the heme nor in the ligands. However, they only report spectra of the ferric proteins between 1000 and 1700 cm^{-1} using 514.5 nm excitation; these conditions are too limited to definitely exclude the possibility of the protein and ligand effects in the resonance Raman spectra. Recently two groups of workers have reported spectra of ferrocyclochromes of *b* and *c* type, which permitted comparison of the heme Raman spectra in different protein environment. Kitagawa and Iizuka [3] compared four *c* type cytochrome using 514.5 nm excitation. We note that in the region between 1000 and 1700 cm^{-1} where they report spectra for all four derivatives, the differences between the derivatives do not exceed 7 cm^{-1} . Adar and Erecinska [4] reported spectra of cytochromes *b* and *b_s*, between 600 and 1700 cm^{-1} using 568.2 and 514.5 nm excitations. Although only small differences in the position of individual bands are observed, they attributed these differences to the protein environment. Woodruff et al. [5] have concluded that the resonance Raman spectra of the hemes in different proteins and solution are not different (by comparing the spectra of Co-protoporphyrin in hemoglobin, myoglobin and piperidine).

We have obtained the resonance Raman spectra of the *c* type cytochrome from *Synechococcus lividus*, a blue-green alga, grown on water and $^2\text{H}_2\text{O}$ media using 441.6 nm excitation to record spectra between 100 and 1700 cm^{-1} for both oxidation states. Although limited work has been made to physically characterize this protein in detail, optical and EPR measurements indicate that it contains a *c* type heme which exists in two forms, one bis-histidine and the other, having an unidentified non-methionine ligand (Hoffman, B. and Crespi, H., unpublished).

Thus Raman measurements on the protein may shed some light on the above-mentioned questions.

MATERIALS AND METHODS

The algal cytochrome was isolated according to the procedure described by Crespi et al. [6] using water throughout the preparation so that only the non-exchangeable hydrogens remain deuterated in the protein isolated from $^2\text{H}_2\text{O}$ -grown alga. Horse heart cytochrome *c* (type VI) was obtained from Sigma and used without further purification.

Resonance Raman spectra were recorded with a system described previously [7] using the 441.6 nm line of a Spectra Physics Model 185 HeCd laser. The laser power used was approx. 25 mW and depolarization ratios were measured by taking spectra at parallel and perpendicular polarization using a polaroid analyzer. All the spectra were taken at 5 °C as described previously [8]. The sample concentration is noted in the figure legends. Reduction was accomplished by addition of a minimal amount of $\text{Na}_2\text{S}_2\text{O}_4$ crystals. Optical absorption spectra were recorded with Zeiss DMR 21 or Cary 14 spectrophotometers to determine the heme concentration.

RESULTS

The spectra obtained with 441.6 nm excitation are shown in Figs. 1 and 2. It should be noted that the signal-to-noise ratios in the low frequency region are particularly good down to 100 cm^{-1} in the reduced protein. On the other hand, we had some difficulty resolving some of the weak bands in the $900\text{--}1300\text{ cm}^{-1}$ region. For example, resolution of the bands at 1339 and 1397 cm^{-1} were made by comparing the unpolarized (Fig. 2) and polarized (Fig. 2, inset) spectra. Some of the bands in this region appear depolarized and relatively strong with excitation in the α and β bands in the spectra of horse heart cytochrome *c* [10, 11]. We have tabulated frequencies and deuterium shifts and for comparison added frequencies of resonance Raman bands of horse heart cytochrome *c* in Table I. As can be seen clearly from the figures, identification of corresponding bands in the $800\text{--}1300\text{ cm}^{-1}$ region between the protonated and deuterated species is difficult. We have measured depolarization ratios of the ferrocytochrome in the entire frequency range and of the ferricytochrome between 100 and 900 cm^{-1} with the hope of using this property in the identification of the shifted bands. However, the number of bands, particularly in the region of difficulty,

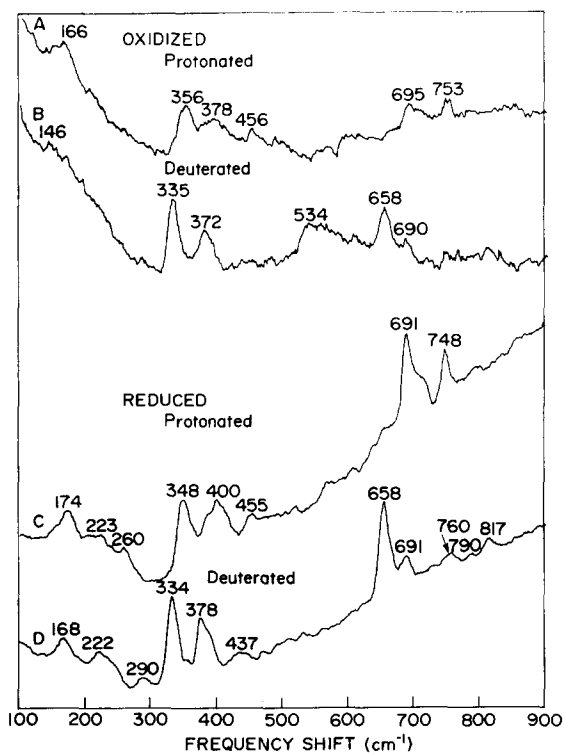


Fig. 1. Resonance Raman spectra of algal cytochrome from *S. Lividus*: low frequency region. Relative system gains for a and c, 3:1, for b and d, 3:1. The heme concentration of the samples: protonated, $107\text{ }\mu\text{M}$ and deuterated, $174\text{ }\mu\text{M}$. Single scans taken in transmission geometry, scan speed, $1\text{ cm}^{-1}/\text{s}$, spectral slitwidth, 5 cm^{-1} , each spectrum was first stored in a multichannel analyzer and read out on an X-Y recorder with an additional time constant of 2–3 s.

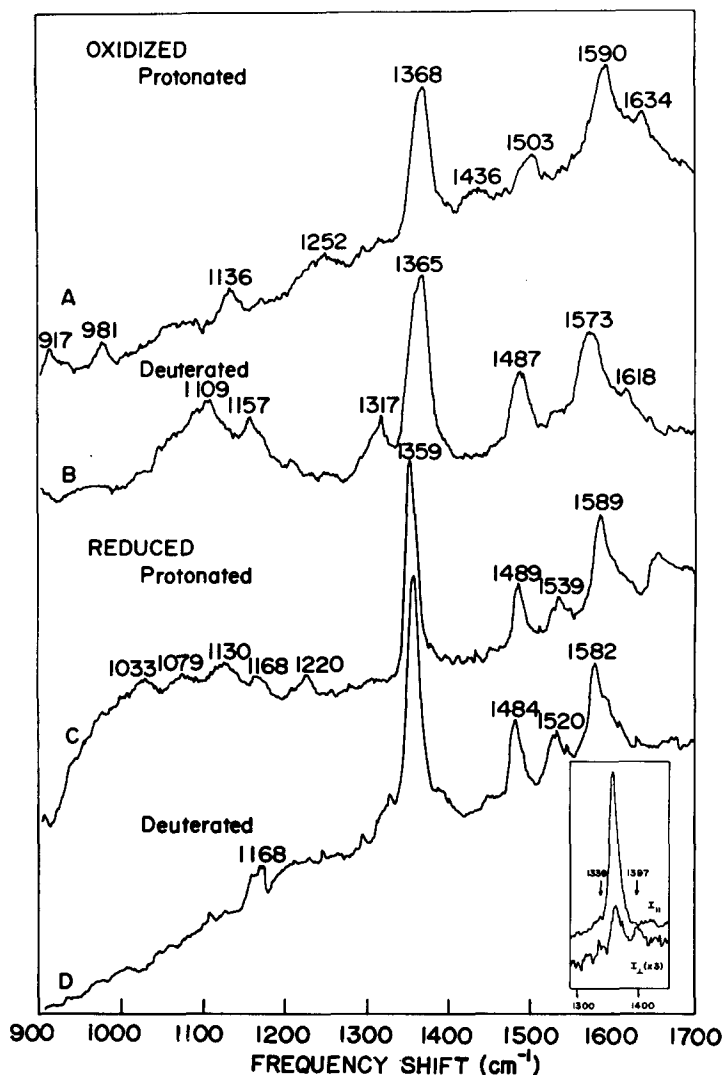


Fig. 2. Resonance Raman spectra of algal cytochrome from *S. Lividis*: high frequency region. Relative system gains, for a and c, 1.5:1.0, for b and d, 3:1. Insert: Polarized spectra for portion of trace c.

was not the same in the normal and deuterated spectra and depolarization ratios were not necessarily the same for the shifted bands, which made it impossible to assign all the bands. Although there have been some assignments of the overtone and combination bands of cytochrome *c* in this region [11, 13, 14], the absence of complete polarization data leads us to restrict the list of deuterium shifts to those bands which could be identified easily.

DISCUSSION

If peripheral substituents contribute to the resonance Raman spectra of algal

TABLE I

RESONANCE RAMAN SPECTRA OF *c* TYPE CYTOCHROME WITH 441.6 nm EXCITATION. POSITIONS OF EXTREMA IN cm^{-1}

Ferricytochrome			Deuterium shift	Ferrocyclochrome			Deuterium shift
Horse***	Algal			Horse***	Algal		
	H	² H			H	² H	
1634 (0.6)**	1634	1618	16	1620 (0.2)	1607 (0.3)	1595 (0.3)	12
*1583 (0.1)	1590	1573	17	1594 (0.1)	1589 (0.3)	1582 (0.2)	7
				1546 (0.2)	1539 (0.5)	1520 (0.7)	19
*1500 (0.2)	1503	1487	16	1491 (0.0)	1489 (0.1)	1484 (0.2)	5
	1436	1317	119				
				1395 (0.3)	1397 (1.0)	1395 (0.3)	2
*1367 (0.2)	1368	1365	3	1359 (0.03)	1359 (0.1)	1359 (0.2)	0
1313 (0.6)				1308 (0.4)	1334 (0.2)	1328 (0.7)	6
1230	1252	1157			1270	1200	
				1228 (0.6)	1220	1187	
1172 (0.6)				1175 (0.3)	1168	1170	
1126 (0.4)	1136	1109		1126 (0.1)	1130	1152	
					1079	1015	
1096						817	
960	980	820		973 (0.3)	968	790	
	917 (?)					760	
755	753 (0.7)	690 (0.6)	63	751 (0.5)	748 (0.6)	691 (0.5)	57
				727	712		
* 702	695 (0.5)	658 (0.3)	37	691 (0.1)	691 (0.1)	658 (0.1)	33
570				568 (0.3)			
485							
450	456			450	455 (0.1)	437 (0.5)	
416				416	416	392 (0.2)	14
400	378 (0.6)	372 (0.4)	6	396 (0.1)	400	378 (0.1)	22
386				374	386		
* 352	356 (0.2)	335 (0.2)	21	355 (0.1)	348 (0.2)	334 (0.1)	14
				305		290 (0.1)	
				264	260	222	
					223	205	
* 153	166	146	20	177	174 (0.5)	168 (0.5)	6

* Tentatively assigned as A_{1g} skeletal modes.

** Depolarization ratio, $\rho_1 = I_{\parallel}/I_{\perp}$.

*** The part of data on horse heart cytochrome *c* taken from Yamamoto [12]. We were unable to calculate depolarization ratios for those bands of ferricytochrome below 1100 cm^{-1} because of poor signal-to-noise ratios with parallel polarization.

cytochrome, then substituent of hydrogen by deuterium in the porphyrin ring should produce some degree of deuterium shift to an extent depending on the type of contribution. If the contributions are due to mixing of substituent and skeletal vibrations, then deuterium shifts may be relatively small, while larger deuterium shifts may be observed in the CH_2 , CH_3 bending region if the contributions are due to those substituent vibrations which are themselves resonance enhanced. Since in heme *c* thioether linkages replace the vinyl groups, we may observe weak resonance Raman bands in

the single bond C-C stretching region attributable to substituent groups and which are sensitive to deuterium.

The magnitude of shifts in different regions do vary as expected: in the 1300–1700 cm^{-1} region they are small and usually less than 20 cm^{-1} . The resonance Raman spectra of normal metalloporphyrins and their *meso*-deuterated derivatives exhibit similar shifts in this frequency range [13, 15, 16] and it appears likely that the deuterium shifts of the heme *c* in algal cytochrome in this region demonstrate only a small amount of mixing of the methine bridge CH deformation or perhaps CH stretching with the porphyrin macrocycle stretching modes (as suggested by Kitagawa et al. [15]).

There are, however, several bands which are very little affected by deuterium substitution. One is the 1360 cm^{-1} band present in both oxidation states of the algal cytochrome and the corresponding bands in various metalloporphyrins. Among the hemoproteins this band has been identified as iron-valence dependent [2, 16, 17]. In contrast, the band at 1580 cm^{-1} which is insensitive to deuterium substitution at the *meso* position in metalloporphyrins exhibits a deuterium shift of 17 cm^{-1} (Fe^{3+}) and 7 cm^{-1} (Fe^{2+}) in the algal cytochrome implying that the 1580 cm^{-1} band contains a small contribution from peripheral substituents.

The vibrations in the 1000–1300 cm^{-1} region are presumed to be single bond C-C stretching or bending modes involving CCC, CCH and HCH bonds. Although we have been unable to assign any deuterium-shifted bands in this region, we take note of the fact that there are no resolved bands in the region 1170 and 900 cm^{-1} , while a few bands appear around 800 cm^{-1} . This suggests substantial deuterium shifts for some of the bands between 1170 and 1300 cm^{-1} and thus involvement of CCH and HCH bending modes. On the other hand, metalloporphyrins exhibit smaller deuterium shifts in this region, which suggests a very small contribution of methine bridge vibrations to the normal modes. Thus the larger deuterium shifts may be due to CCH, HCH bending modes in the peripheral substituents or else the normal coordinates for the algal heme *c* and those metalloporphyrins studied are quite different. In the low frequency region, the bands at approx. 750, 690 and 350 cm^{-1} are considered to be fundamentals while the other bands may be difference combination bands [11]. The significant shifts observed in the low frequency region can be attributed to an appreciable degree of mixing between the skeletal vibrations and substituent or *meso* CH vibrations. Among the metalloporphyrins studied, only the Cu- and Fe-porphyrin data are adequate for comparison in this region. In general, the two metalloporphyrins exhibit smaller deuterium shifts than algal cytochrome with the exception of a depolarized band at 751 cm^{-1} which appears at 690 cm^{-1} in the deuterated Cu compound; this is comparable to the deuterium shifts observed for this band in algal cytochrome.

A normal coordinate analysis is required to determine to what degree the methine bridge or substituent vibrations contribute to the skeletal modes which appear strong in the resonance Raman spectra of heme proteins. This would be a complex task if all the substituents were to be taken into account (Yamamoto, T. and Taylor, R.C., unpublished and ref. 1).

With a few exceptions, the oxidized cytochrome exhibits somewhat larger shifts than the reduced protein. For example, the bands at 1590, 1503, 695, 356 and 166 cm^{-1} in the oxidized protein exhibit deuterium shifts of 17, 16, 37, 21, 20 cm^{-1} , respectively, while in the reduced protein the corresponding bands show deuterium

shifts of 7, 5, 33, 14, and 6 cm^{-1} (the bands marked with asterisks except the 1360 cm^{-1} band in Table I); this may signify conformational changes in the porphyrin on reduction.

In comparing horse heart and algal cytochromes we find that the polarized spin-state-dependent band at 1580–1590 cm^{-1} which shifts on reduction of the heme in the mammalian protein [16], hardly shifts in the algal cytochrome upon reduction. A second characteristic band of horse heart cytochrome *c* at 702 cm^{-1} also shifts upon reduction, to 691 cm^{-1} . In algal cytochrome, the difference between the bands in two oxidation states is only 4 cm^{-1} , within experimental uncertainty. Furthermore, we note that horse cytochrome *c* exhibits a band at 568–570 cm^{-1} in both oxidation states while no comparable bands have been observed with algal cytochrome.

While heme *c* or mesoheme exhibits an inversely polarized band at 1315 cm^{-1} protoheme exhibits two bands at 1305 and 1340 cm^{-1} in the reduced proteins [4, 5, 7]. With 441.6 nm excitation, we observe a polarized band at 1308 cm^{-1} in the spectrum of horse ferrocytochrome *c*. In the spectrum of algal ferrocytochrome (Fig. 2c and insert) we observe a polarized band at 1339 cm^{-1} *: in the oxidized state, horse cytochrome *c* exhibits a band at 1313 cm^{-1} ($q = 0.6$), but this is not observed in the algal cytochrome.

Despite the clear demonstration by Woodruff et al. [5] that neither the protein nor ligand has any effects on the Raman frequencies of the heme, it is still conceivable that the protein or ligand effects exist in *c* type cytochromes in which the heme is bound covalently to the protein and presumably more constrained than in protoheme proteins. The differences we observe between the two *c* type proteins can be due to either ligand or protein effects or both.

Since the Soret bands of horse cytochrome are 410 nm for oxidized and 416 nm for reduced protein, we cannot completely rule out possibilities that some Raman bands are hidden, particularly in the spectrum of oxidized algal cytochrome obtained with the 441.6 nm excitation used for this study. Nevertheless, most of the observed differences between the two cytochromes must be independent of how close the Soret bands are to the excitation frequency and can be attributed to the differences in the heme in these two *c* type cytochromes.

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* There is a small feature present at 1310 cm^{-1} but the quality of the data precludes a secure assignment.

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