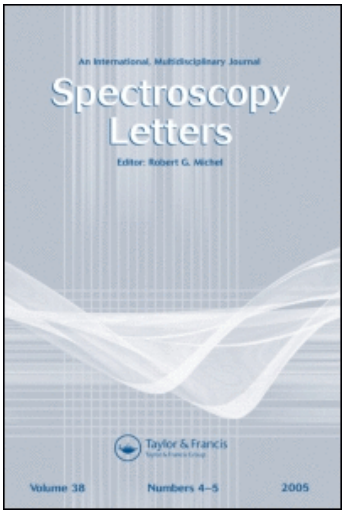

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FTIR STUDY OF THE SECONDARY STRUCTURE OF CYTOCHROME *C* AND ITS PLATINUM-MODIFIED DERIVATIVES

Key Words: FTIR, secondary structure, platinum, cytochrome *c*

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ABSTRACT

The FTIR spectral measurements were carried out for native cytochrome *c* (cyt *c*) and its four platinum-modified derivatives. The influence of the platinum complex binding on the secondary structure of cyt *c* was discussed. It was found that the secondary structure of platinum-binded derivatives is similar to that of native cyt *c* when the platinum complex is binding on or near the surface of the protein. While in the case of derivatives, in which the platinum complex interacts with the second axial ligand of cyt *c* (Met 80) and causes the replacement of Met 80 by Lys 79 or solvent (H_2O), great secondary structural changes were observed.

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The results imply that the Met 80 residue is very important in controlling the secondary structure of cyt *c*.

INTRODUCTION

The platinum complexes such as *cis*- and *trans*-dichlorodiamine are known to interact with sulfur containing ligands, for example glutathione, metallothionein *etc. in vivo* and *in vitro*.[1] We have found that there is also interaction between such platinum complexes and electron transfer protein as cytochrome *c* (cyt *c*).[2] In the previous paper, the interaction of cyt *c* with *trans*-$PtCl_2(NH_3)_2$ (*trans*-Pt) under different conditions was reported.[2] The results indicate that the *trans*-Pt can bind to not only the surface of cyt *c* (His 33 and Met 65 residues) but also the axial ligand (Met 80 residue), and causes the Fe-S bond to be broken and a facile spin conversion of Fe^{3+} (from low spin to high spin).[2] Four kinds of platinum-modified cyt *c* derivatives were obtained at pH 7.0, they are singly labeled Pt-His 33 (cyt *c*) in which *trans*-Pt binds to His 33 through the N of imidazole ring; singly labeled Pt-Met 65 (cyt *c*) in which the binding site of platinum is thioether of Met 65 residue; doubly labeled Pt-His 33 & Met 65 (cyt *c*) in which *trans*-Pt binds to both His 33 and Met 65 residues; and multiply labeled low-spin state cyt *c* dimer Pt-(Met 80-cyt *c*-Lys 79)$_2$ (Pt/Fe molar ratio of 5:2) in which not only His 33 and Met 65 residues of each cyt *c* molecule may be coordinated by *trans*-Pt but also the proteins are cross-linked by *trans*-Pt through the S atoms in their respective Met 80 residues and the second axial ligand of cyt *c* is replaced by Lys 79. At pH 5.5, besides singly labeled Pt-His 33 (cyt *c*) and Pt-Met 65 (cyt *c*), a multiply labeled high-spin state cyt *c* dimer Pt-(Met 80-cyt *c*-H_2O)$_2$ (Pt/Fe molar ratio of 7:2) was also obtained. In this case not only His 33 and Met 65 and His 26 residues of each cyt *c* molecule may be coordinated by *trans*-Pt but also the proteins are cross-linked by *trans*-Pt through the S atoms in their respective Met 80 residues and the second axial ligand is replaced by H_2O at pH 5.5.

On the other hand, the FTIR (fourier transform infrared) spectroscopy provides a very practical approach to determination the secondary structure of

proteins. The amide I band due to the C=O stretch vibrations of peptide linkages between 1700 to 1600 cm^{-1} is sensitive to the protein secondary structure.[3-7] The secondary structure of cyt *c* in solution has been investigated by FTIR.[6] We measured the IR spectrum of cyt *c* in solid state and the comparison of the secondary structure of cyt *c* between solid and solution was carried out. Furthermore, in order to understand the influence of *trans*-Pt complex binding on the secondary structure of cyt *c*, we report herein the secondary structures of several above mentioned *trans*-Pt-modified cyt *c* derivatives determined by self-deconvolved FTIR and curve-fitting procedures. A significant difference between the structure of derivatives, in which the Met 80 is replaced by Lys 79 or H_2O due to the binding of the platinum complex to the Met 80, and that of native cyt *c* was observed. Our FTIR studies indicate that the axial ligand of Met 80 residue plays an important role in controlling the secondary structure of cyt *c*.

EXPERIMENTAL SECTION

Sample Preparation

Cyt *c* from horse heart (type VI) from Sigma Chemical Co. was purified prior to use.[8] The *trans*-Pt-modified cyt *c* derivatives were synthesized as described previously.[2] Four kinds of platinum-modified cyt *c* derivatives were obtained and characterized by NMR , UV-vis spectra and magnetic susceptibility as reported in reference 2. These four kinds of platinum-modified cyt *c* derivatives are singly labeled Pt-His 33 (cyt *c*), singly labeled Pt-Met 65 (cyt *c*), multiply labeled low-spin state cyt *c* dimer Pt-(Met 80-cyt *c*-Lys 79)$_2$ (pH 7.0) and multiply labeled high-spin state cyt *c* dimer Pt-(Met 80-cyt *c*-H_2O)$_2$ (pH 5.5), respectively.

All the FTIR spectral samples (except for the cyt *c* solution in H_2O) were dialyzed in D_2O by ultrafiltration repeatedly to exchange all the labile protons. Protein solutions ranging from 3 to 5 % (w/v) were prepared in D_2O and were assembled between CaF_2 windows separated with 50 μm Teflon spacer. The solid cyt *c* was measured as KBr pellets.

IR Spectral Measurements

Infrared spectra were recorded on a Nicolet 170SX FTIR spectrophotometer at room temperature. The instrument was purged with dry air overnight prior to the measurements. Buffer spectra were taken first followed by spectra of protein solutions. The sample chamber was purged during the measurements. For each spectrum, a 1200-scan interferogram was collected at single beam mode with a 2 cm^{-1} resolution from 4000 to 1000 cm^{-1}. Differential spectra were obtained by subtraction of the buffer blank and gaseous water from the protein spectra following the procedures of Dong et al.[9] Second-derivative spectra were obtained by taking the first derivative twice and were used to verify the peak assignments of the deconvolved spectra.[4] Fourier self-deconvolution of the amide I bands was performed by using a Lorentzian of 13 cm^{-1} half-bandwidth and a resolution enhancement factor (k value of 2.4), which give optimal resolution enhancement without excessive deconvolution such as side lobes or other artifacts.[6] Iterative fitting of Gaussian curves to the deconvolved spectra was carried out, and the relative contents of different types of secondary structure were obtained by integrating the area under the curves assigned to a particular configuration.

RESULTS AND DISCUSSION

The FTIR spectra of cyt *c* in H_2O and D_2O solution are shown in Figure 1A. Infrared spectra of proteins in H_2O typically show two bands, one between 1700 and 1600 cm^{-1} and one between 1600 and 1500 cm^{-1}. The former is the amide I band, and the latter is a complex of the amide II band and bands due to side-chain vibrations. Both amide bands are complex composites of several discrete bands that are characteristic of specific types of secondary structure of the protein. In D_2O solution the accessible N-H groups will undergo H-D exchange and there will be a small shift of the broad amide I bands and a large shift of the amide II bands. As seen in Figure 1A, the shift of the 1650 cm^{-1} band between the two solvents indicates that extensive exchange of H for D has occurred. This is supported by the large observed decrease in intensity of the amide II band centered at around

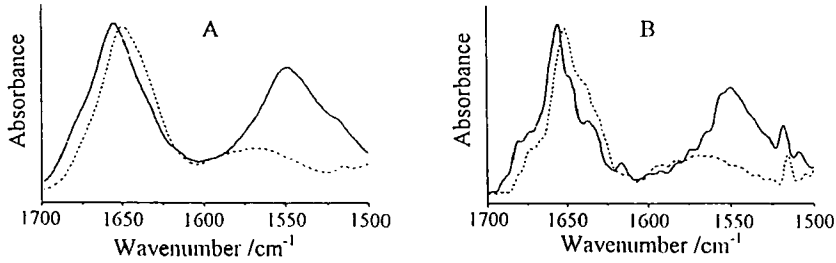

Fig. 1. Infrared spectra of cyt *c* in H_2O and D_2O in the region of the amide I and amide II (1700 -1500 cm^{-1}). (A): the original infrared spectra and (B): the spectra after band enhancement by Fourier self-deconvolution. (—)sample in H_2O buffer solution; (---) sample in D_2O buffer solution.

1550 cm^{-1}. The peak that still remains in the spectrum in D_2O at 1570 cm^{-1} is attributed to COO^- groups of Asp and Glu that cyt *c* contains.[10]

More information can be obtained from the rather featureless amide I bands by a process of Fourier self-deconvolution. It was performed to produce the curves shown in Figure 1B, where it can be seen that each of the component bands of the amide I bands is shifted by approximately 5 cm^{-1} to lower frequencies in D_2O compared with the corresponding bands in H_2O. This shift occurred within 24 hours, and no further shift was observed with longer times. In the deconvolved spectrum of the D_2O sample, it can also be seen that the actual absorbance at 1550 cm^{-1}, due to the amide II band, is very small, again confirming that there has been extensive H-D exchange.

The quantitative contribution of each band to the total amide I contour can be obtained by the curve-fitting procedures. Figure 2 shows the deconvoluted spectra and the fitted components for the amide I bands of solution and solid cyt *c* and their second derivative spectra. It can be seen from Figure 2 that the number and wavenumber of the peaks in the second-derivative spectra are consistent with the corresponding value in the deconvolved spectra. This indicates that our results are reliable. Eight bands between 1700 and 1620 cm^{-1} are considered to be due to

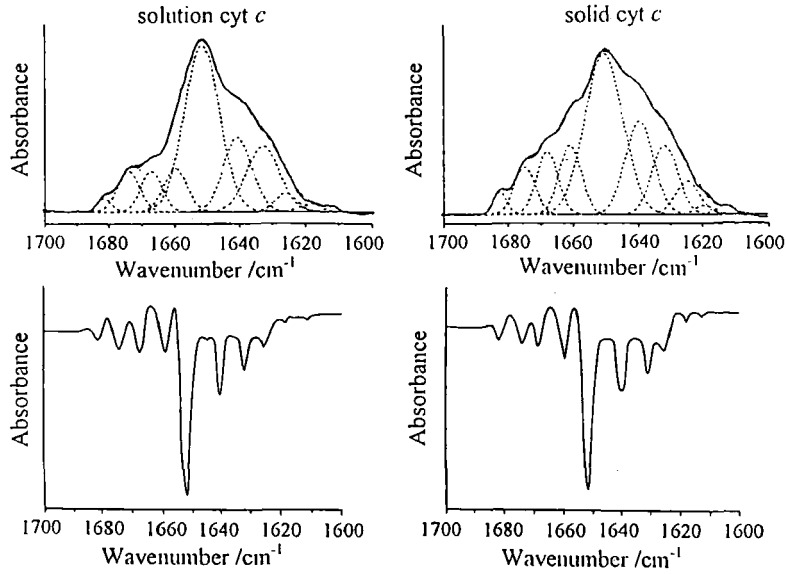

Fig. 2. Deconvolved spectra and fitted amide I components for solution and solid cyt *c* (upper curves), and their second derivative spectra (lower curves). In the upper curves, solid lines represent the experimental deconvolved spectrum; the dotted lines give the resolved components and the dash lines show the calculated sum of the resolved components (Because the deviation between the calculated curve and the experimental data is so small, the former is virtually superimposed on the experimental spectrum).

the vibrations of the amide I bands, and the two bands below 1620 cm^{-1}, although included in the curve-fitting procedure, are due to side-chain vibrations.[10]

The amide I band assignments for secondary structures available from previous studies[6,9] are as follows: α-helix (1650±2 cm^{-1}), β-extended chain (multiple bands between 1635 and 1620 cm^{-1}), unordered (1640±2 cm^{-1}) and turns (multiple bands between 1685 and 1665 cm^{-1}). The wavenumber of each type of above substructure in D_2O is lower by approximately 5 cm^{-1} than the corresponding value in H_2O because each of the component bands of the amide I bands is shifted to lower frequencies in D_2O. However, the assignment of the

band at 1660 cm^{-1} in the deconvolved spectrum is more difficult. The high-resolution crystallographic study shows that there are six β-turn structures in the oxidized horse heart cyt *c*: two 3_{10} helices (type III turns) and four type II turns.[11] These two 3_{10} helices are located at residues 14-17 and 67-70, respectively, where they make turns to connect the β-extended chains and α-helices. Because the 3_{10} helix is less common, it has been less well studied by infrared spectroscopy. Synthetic α-aminobutyric acid-containing peptides, which are known to form 3_{10} helices, show strong amide I bands at 1662-1663 cm^{-1}.[12] Other experimental and theoretical studies also indicate that distorted helical structures such as 3_{10} or α_{II}-helices exhibit higher amide I frequencies than those observed for α-helices due to weaker hydrogen bonding.[12-14] Therefore, we can reasonably assign the 1660 cm^{-1} band to the 3_{10} helices structure.

The deconvolved spectra were subjected to curve fitting, and the quantitative contribution of each type of substructure to the total amide I band contour may be obtained by integrating band areas assigned to each substructure.[6,7] Table 1 presents the observed frequencies, vibrational assignments and relative area for each amide I band component of cyt *c* in solution and solid state. In Figure 2 and Table 1, the most prominent band at 1650 cm^{-1}, which has been assigned to α-helical structure, confirmed that the α-helix is the most abundant type of secondary structure of cyt *c* in both solid and solution state. Quantitative evaluations (shown in Table 1) indicate that both solid and solution cyt *c* consist of 40-44% α-helix, 17-18% β-extended chain, 7-10% 3_{10} helix, 15-16% β-turns and 16-17% unordered structure. These results agree well with the values calculated from high-resolution x-ray structure of oxidized horse heart cyt *c* and previous FTIR study,[6,11,15,16] which again suggest our results are reliable. From Table 1 and Figure 2, it can be seen that the solid structure of cyt *c* is similar to its solution structure, which further confirms the general similarity of solution and crystal structure for many proteins.[6]

TABLE 1
Amide I band components observed in the deconvolved FTIR spectra of cyt *c*.

Sample	Frequency (cm^{-1})	Assignment	Content (%)
Solution cyt *c*	1682, 1674, 1667	β-turn	15
	1660	3_{10}-helix	7
	1651	α-helix	44
	1641	unordered	16
	1632, 1625	β-extended chain	18
Solid cyt *c*	1682, 1675, 1667	β-turn	16
	1661	3_{10}-helix	10
	1651	α-helix	40
	1641	unordered	17
	1632, 1625	β-extended chain	17
Crystal cyt *c*[a]		β-turn	15
		3_{10}-helix	8
		α-helix	45
		unordered	17
		β-extended chain	15

[a] Data from References 11, 15, 16.

The secondary structure of platinum-modified cyt *c* derivatives were also determined by FTIR spectroscopy. Figure 3 shows the deconvolved spectra and the fitted components for the amide I bands of four platinum-modified cyt *c* derivatives. The deconvolved spectra were subjected to curve fitting, and the percentage of each type of substructure content are presented in Table 2. The secondary structures of two kinds of Pt-modified cyt *c* derivatives *trans*-Pt-His 33 (cyt *c*) and *trans*-Pt-Met 65 (cyt *c*) are similar to that of native cyt *c* (Table 2). The results suggest that the binding of platinum complex to His 33 and Met 65 residues which are on or near the surface of cyt *c* do not cause significant secondary structural changes of the protein.

However, there are substantial differences between the secondary structures of low- or high-spin cyt *c* dimers Pt-(Met 80-cyt *c*-Lys 79)$_2$, Pt-(Met 80-cyt *c*-H_2O)$_2$ and that of native cyt *c*, especially for the α-helix and unordered substructure components (Table 2). This is considered to be caused by

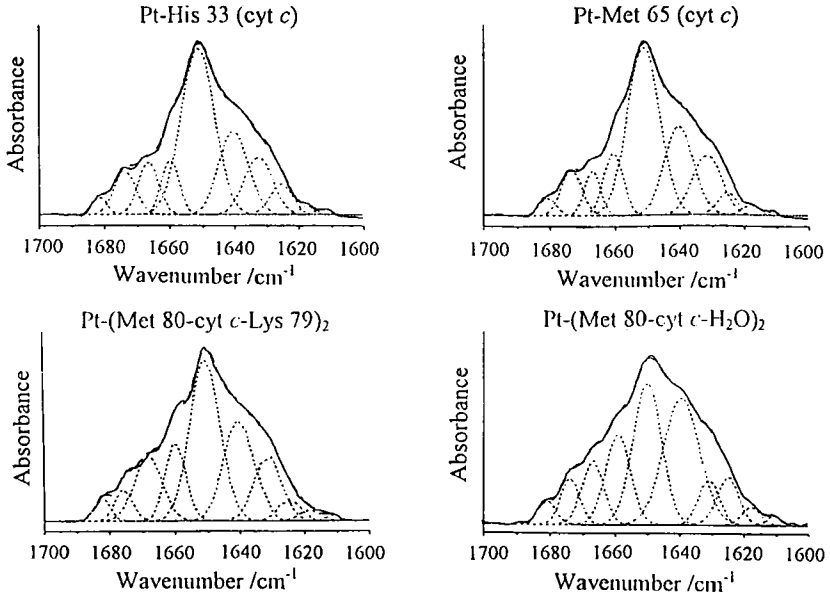

Fig. 3. Deconvolved spectra and fitted amide I components of platinum-modified cyt *c* derivatives. In the curves, solid lines represent the experimental deconvolved spectrum; the dotted lines give the resolved components and the dash lines show the calculated sum of the resolved components (Because the deviation between the calculated curve and the experimental data is so small, the former is virtually superimposed on the experimental spectrum).

replacement of second axial ligand (Met 80) by non-native Lys 79 or H_2O supplied by solvent due to the binding of platinum complex to the Met 80 of cyt *c*. Compared with the structure of native cyt *c*, it can be seen that the increase of unordered structure content was at the expense of α-helix content in Pt-(Met 80-cyt *c*-Lys 79)$_2$ and Pt-(Met 80-cyt *c*-H_2O)$_2$, *i.e.*, the molecular structures of these two derivatives become more random. The results suggest that the axial Met 80 residue plays an important role in stabilizing the secondary structure of cyt *c*. However, these two platinum-modified derivatives are not fully unfolded and they adopt a compact, molten globule-like state containing substantial secondary structure which is similar to the intermediates in the cyt *c* folding process.

TABLE 2
Amide I band components observed in the deconvolved FTIR spectra of platinum-modified cyt *c* derivatives.

Sample	Frequency (cm^{-1})	Assignment	Content (%)
Pt-His33(cyt *c*)	1682, 1674, 1667	β-turn	17
	1660	3_{10}-helix	7
	1651	α-helix	41
	1640	unordered	18
	1632, 1625	β-extended chain	17
Pt-Met65(cyt *c*)	1681, 1674, 1667	β-turn	15
	1661	3_{10}-helix	9
	1651	α-helix	40
	1640	unordered	20
	1632, 1625	β-extended chain	16
Pt-(Met80-cyt *c*-H_2O)$_2$	1681, 1674, 1667	β-turn	17
	1660	3_{10}-helix	14
	1650	α-helix	26
	1640	unordered	30
	1631, 1625	β-extended chain	13
Pt-(Met80-cyt *c*-Lys79)$_2$	1682, 1675, 1668	β-turn	18
	1660	3_{10}-helix	11
	1651	α-helix	33
	1640	unordered	22
	1631, 1625	β-extended chain	16

Roder et al. reported that the N- and C-terminal α-helices of cyt *c* form during the early folding phase on the 10 ms time scale and the ligation of Met 80 to the heme iron does not interfere with the docking of the N- and C-terminal helices.[17] According to the foregoing conclusion, N- and C-terminal α-helices should be retain in the high-spin platinum-modified cyt *c* derivative Pt-(Met 80-cyt *c*-H_2O)$_2$ and its α-helix content should be 28% although its Fe-S bond is broken. Our FTIR studies show that the α-helix content (26%) of Pt-(Met 80-cyt c-H_2O)$_2$ is close to the above value (28%), which further confirm our method is reliable and the ligation of Met 80 does not interfere with the formation of the N- and C-terminal helices of cyt *c*.

With the spin-state conversion, the α-helix content (33%) of low-spin platinum-modified cyt *c* derivative Pt-(Met 80-cyt *c*-Lys 79)$_2$ is higher than that of high-spin Pt-(Met 80-cyt *c*-H_2O)$_2$. However, its α-helix content (33%) is still lower than that of native cyt *c* (44%) because its non-native axial ligand Lys 79 is likely to prevent the formation of correctly folded structure in other parts of the molecule, including the loop region spanning residues 20-60, as well as the 60's and 70's that are close contact with Met 80 in the native structure.[17]

CONCLUSION

The FTIR method is believed to be suitable for the studies of secondary structural changes. In this paper, we measured the IR spectra of native cyt *c* in both solid and solution states and four platinum-modified cyt *c* derivatives. Their secondary structural comparison was carried out. It is found that the solution structure of cyt *c* is similar to its solid structure. While in the cases of platinum-modified derivatives, the secondary structures are dependent on the binding site of *trans*-Pt complex. When the interaction between *trans*-Pt complex and cyt *c* only occurred in the surface of cyt *c* (His 33, Met 65 *etc.*), no much secondary structural change was observed. However, when the *trans*-Pt complex coordinated with Met 80 which is the second axial ligand of native cyt *c*, the secondary structure of cyt *c* changed greatly. The results are considered to be important for studying the cyt *c* folding.

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