The Characterization and Ultraviolet Absorption (UV), Fluorescence (FS), Circular Dichroism (CD), and Raman Spectroscopy of Metallothionein from Liver of Hedgehog Induced by Cadmium

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ABSTRACT

With Sephadex G-50 and DEAE Sepharose Fast Flow column we obtained five isoforms of metallothionein (MT) from liver of hedgehog induced by cadmium (MT-fI, MT-I, MT-fII, MT-II and MT-pII). All the five MTs from hedgehog liver had ultraviolet (UV) absorption shoulder near 250 nm, which disappeared when pH decreased to 1.5. With excitation at 282 nm the fluorescence (FS) emission spectra of the five MTs had a maximum emission (Pm) near 340 nm, and with the pH decreasing the red shift and the quantum yield decreasing could be observed. Circular dichroism (CD) spectra showed that above pH 2.0 there were a strong positive ellipticity band located near 260 nm and a negative band located near 210 nm for the five MTs and a positive band located near 230 nm for MT-fI and MT-II. Raman spectra showed that five main bands were evidently observable near 680, 1320, 1440, 1670 and 2930 cm^{-1} for the hedgehog MTs, but the former four bands were not evidently observable for the hedgehog apo-MTs.

Key words: Hedgehog; Metallothionein (MT); Ultraviolet (UV); Fluorescence emission (FS); Circular dichroism (CD); Raman

Abbreviation: UV, ultraviolet absorption; FS, fluorescence; CD, circular dichroism; MT, metallothionein; Tris, Tris(hydroxymethyl)aminomethane

INTRODUCTION

Metallothionein (MT) is an ubiquitous class of low molecular weight and cysteine-rich proteins binding unusually high amounts of metal ions, such as Cd, Cu, Hg and Zn. One third of the amino acids found in MT from mammalian are cysteines. The location of these cysteines in metallothionein molecules is highly conserved between different species, which suggests that the cysteine residue location is extremely important to the function of MT. MT is thought to play an important role in the homeostasis of metals such as zinc and copper [1], to be involved in the detoxification of heavy metals, and scavenge free radical [2]. The synthesis of MT is induced by various factors such as heavy metals, coldness, heat, hunger, radiation and diseases [3]. Since the discovery of MT by Margoshes and Vallee in 1957 [4], it has been attracted more and more attention in its biological function and medical application. The optical spectra of metallothionein are related to the MT's structure and function [5, 6, 7]. There are some research results for optical spectra of rabbit MTs [5], but no reports published for hedgehog MTs.

MATERIALS AND METHODS

1. Materials

Thirty-six hedgehogs (*Erinaceus eurapaceus*) were caught in the western suburban district of Beijing, China. Twenty-one hedgehogs were male and the other fifteen were female. Average weight of the hedgehogs was 2.1 kg and age of them was not clear; Sephadex G-50, Sephacryl S-100 and DEAE Sepharose Fast Flow were from Parmacia (Sweden); UV spectrophotometer is Beckman DU 7500 (USA); Fluorescence spectrometer is MPF-66 (Perkin-Elmer, Japan); CD spectrometer is Jasco J-500/DP-500 (Japan); Raman spectrometer is IFS 66+FRA 106 (Brucker Co., Switzerland); Ultrapure water (≥17.5 MW, pH 5.4) was obtained from a Milli-Q water system (Millipore Corp., Japan) (All the water used in this experiment was ultrapure water).

2. Methods

1) MT was prepared according to the following procedure [8]: Hedgehogs were injected by $CdCl_2$ (administrated intramanuscularly by 50 mg/kg • wt of $CdCl_2$ within 15 days for five 3-days intervals with the dosages of 5, 10, 10, 10, 15 mg/kg • wt). The liver of hedgehog was removed, homogenized and centrifuged (22,000×g for 30 min at 4°C). The supernatant was heated at 80°C for 5 min, centrifuged, then the supernatant was precipitated by adding ethanol for 3 times volume, over night at -20°C, centrifuged, and the

precipitate was dissolved in 0.02 mol/l Tris-HCl, pH 8.6, and was centrifuged again. The supernatant was separated with gel filtration (Sephadex G-50), ion-exchange column (DEAE Sepharose Fast Flow), then desalted with gel filtration column chromatography (Sephacryl S-100).

2) Mercapto group detection: A 100 ml sample was added into a polarographic cell containing 2 ml of supporting electrolyte (0.1 mol/l NH_4Cl, 0.1 mol/l NH_3H_2O, 2 $\times 10^{-4}$ mol/l $CoCl_2$). Analysis was performed by scanning from -0.05 V to -1.8 V for 2 s using a dropping mercury electrode. The reference electrode is silver electrode. The first derivative of peak current at -1.45 V (vs. SCE) was recorded.

3) Amino acid analysis: Each sample was hydrolyzed at 110°C with 0.5 ml of 5.7 mol/l HCl in vacuum for 24 hours. The dried hydrolysates were then dissolved in 1 ml of 0.2 mol/l sodium citrate acid buffer, at pH 2.2. Another aliquot was oxidized by performic acid at -10°C for 4 hours. The amino acid compositions were determined with a Beckman 121 MB amino acid analyzer.

4) UV absorbance measurement: MT sample was dissolved in 1 ml of 0.01 mol/l Tris-HCl, pH 8.6 buffer, and was scanned from 300 to 190 nm. Then 30 µl 1.2 mol/l HCl was added to the sample solution (to pH 1.0) and scanned again at the same range of wavelength.

5) Fluorescence spectra analysis: MT sample was dissolved in ultrapure water (pH 5.4), and adjusted to pH 3.5, 2.8, 2.0 1.5 and 1.0 with HCl, respectively. The sample solutions were exited at 282 nm and scanned from 320 nm to 400 nm under 20°C.

6) CD spectra analysis: MT sample was dissolved in ultrapure water and adjusted to pH 3.0, 2.0, 1.0 with HCl, respectively, then scanned from 200 to 300 nm, repeated eight times. Contents of α-helix, β-sheet, β-turn and unordered structure in apo-MT was calculated according to the method of Provencher and Glochner [9].

7) Raman spectra analysis: 100 mg MT powder was added into sample chamber and measured under output powder of 200 mw.

RESULTS

With Sephadex G-50 then DEAE-Sepharose Fast Flow columns we obtained five isoforms of MT from liver of hedgehog induced by cadmium, and we named them MT-fI, MT-I, MT-fII and MT-pII, successively (Fig.1). The hedgehog MTs are composed of about 61 amino acid residues, and among them there are 30% of cysteine but lack aromatic and histidine residues (Table 1). There is no sequence of hedgehog MT reported up to now. There were high absorbance at 254 nm and mercapto group peaks at the MT fractions of DEAE column. The total metal content in MT molecule (Cd+Zn) was 7 and the ratio of Cd to Zn of the five isoforms (MT-fI, MT-I, MT-fII, MT-II and MT-pII) were 2.30, 3.62, 1.91, 3.00 and 1.36, successively.

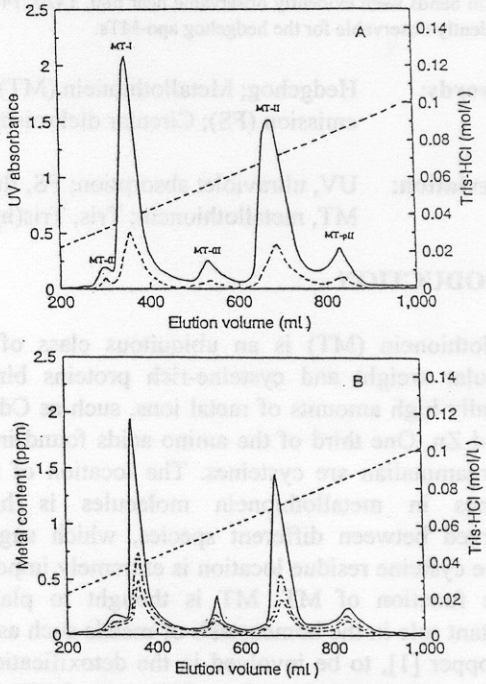

Fig. 1 Ion-exchange chromatography of Cd-induced hedgehog liverMTs on DEAE-Sepharose Fast Flow column. Column size: 2.6×18 cm. Eluted with: A) 0.01 mole/L Tris-HCl, pH 8.6, 1000 ml; B) 0.2 mol/L Tris-HCl, pH 8.6, 1000 ml. Flow rate: 1.5ml/min. A: O.D. 254 nm (——); O.D. 280 nm (- - - -). B: Cd (——); Zn (- - - -); Mercapto group (mA)(- · - · - · -)

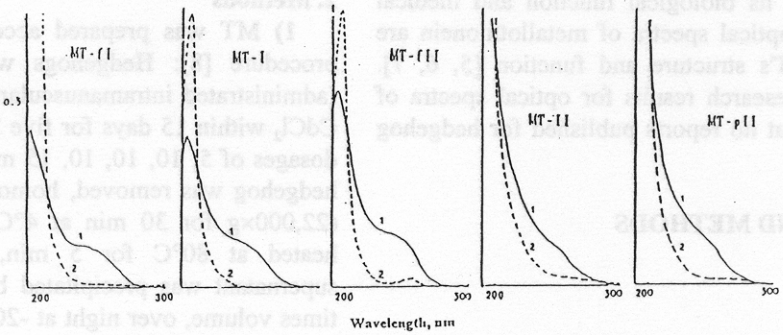

Fig. 2 Ultraviolet absorption spectra of Cd-induced hedgehog liver MTs. 1: Sample (50 mg/mL) 2 ml in 0.01 mol/L Tris-HCl, pH 8.6; 2: 1 added 30 ml 1.2 mol/L HCl

From Fig.2 we can see that all the five MT isoforms had an absorption shoulder at 250 nm, which is the characteristic absorbance of Cd(II)-SH.

Table 1 Amino acid composition of hedgehog MTs

aa	MT-f I		MT- I		MT-f II		MT- II		MT-p II	
	mol%	aa/M	mol%	aa/M	mol%	aa/M	mol%	aa/M	mol%	aa/M
Cys	28.69	17.60(18)	30.29	18.48(18)	30.00	18.30(18)	27.36	16.68(17)	28.22	17.21(17)
Asx	6.79	3.63(4)	6.36	3.88(4)	6.18	3.77(4)	6.50	3.97(4)	9.60	5.86(6)
Thr	3.87	2.36(2)	4.06	2.48(2)	4.08	2.49(2)	3.19	1.96(2)	4.36	2.66(3)
Ser	8.33	5.08(5)	11.18	6.82(7)	10.20	6.22(6)	15.20	9.27(9)	10.49	6.40(6)
Glx	8.06	4.91(6)	5.06	3.08(3)	4.17	2.54(2)	4.88	2.98(3)	7.26	4.42(4)
Pro	8.88	5.42(5)	5.96	3.64(4)	3.08	1.88(2)	7.47	4.56(4)	4.23	2.58(2)
Gly	8.92	5.44(6)	6.89	4.20(4)	8.18	4.99(5)	7.68	4.62(5)	8.64	5.21(6)
Ala	9.28	5.66(6)	10.82	6.60(6)	9.78	5.97(6)	8.14	4.97(5)	12.78	7.80(8)
Val	0.53	0.32(0)	0.73	0.45(1)	2.00	1.22(1)	3.46	2.10(2)		0
Met	1.84	1.12(1)	3.02	1.84(2)	2.73	1.67(2)	2.62	1.54(2)		0
Ile	1.67	0.96(1)	0.95	0.57(1)	3.01	1.84(2)		0	4.60	2.81(3)
Leu	0.94	0.57(1)	0.67	0.41(1)	0.67	0.36(1)		0	2.72	1.66(2)
Tyr		0		0		0		0		0
Phe		0		0		0		0		0
Lys	12.32	7.52(8)	13.06	7.96(8)	14.99	9.14(9)	13.72	8.37(8)	10.49	6.40(6)
His		0		0		0		0		0
Arg	0.99	0.60(1)	0.97	0.59(1)	1.0	0.63(1)		0	0.41	0.26(0)
Total		(62)		(62)		(61)		(61)		(62)

According to the Fig.3 of fluorescence spectra, when excited at 282 nm all the five hedgehog MTs had maximum emission near 340 nm (at pH 5.4, Pms of the five MTs were 345.1, 332.9, 338.6, 336.1 and 344.1 nm, successively). With the decrease of pH, the Pms of all the five MTs made red shift and quantum yield drop considerably. The order of the Pm wavelength for the five MTs was MT-fI>MT-pII>MT-fII>MT-II> MT-I, which is converse to the ratios of Cd to Zn of MTs, i.e. the more the cadmium MT contained, the shorter the wavelength of Pm it had.

Fig.4 was the CD spectra of the five hedgehog MTs at different pH value. From the figure it could be seen that above pH 2.0 all the five MTs had strong positive absorption bands near 260 nm and negative absorption bands near 210 nm. For MT-fI, MT-fII and MT-pII there were positive absorption bands near 230 nm. With pH declining, the positive band near 260 nm for all the MTs diminished gradually, while the negative band near 210 nm became stronger.

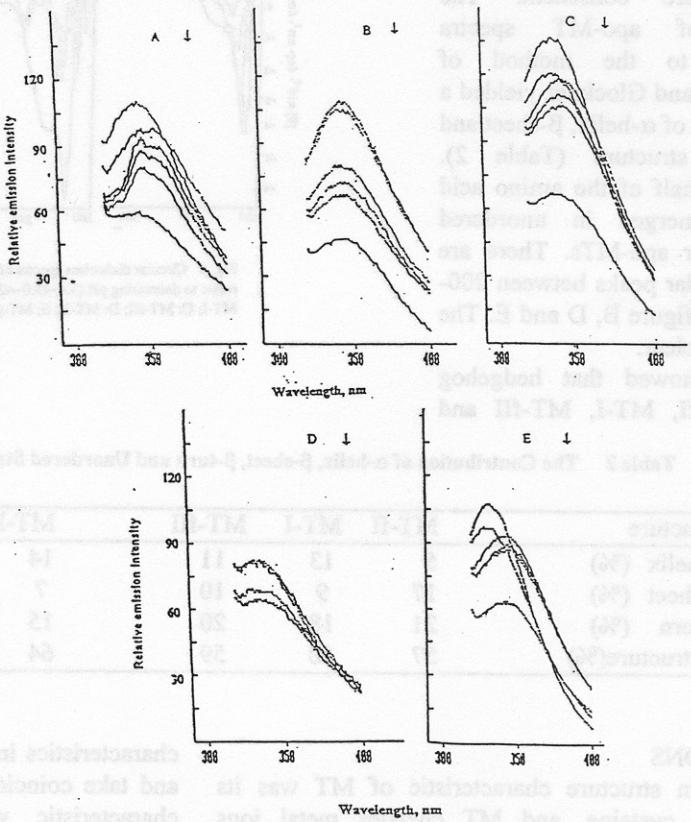

Fig. 3 Fluorescence emission spectra of Cd-induced hedgehog MTs. The arrows relate to decreasing pH (5.4→3.5→2.8→2.0→1.5→1.0). Sample 2 ml, 50 mg/mL. A: MT-fI; B: MT-I; C: MT-fII; D: MT-II; E: MT-pII

Under strong acid condition of pH 1.0, each hedgehog MT had a very strong negative absorption band near 210 nm, while the positive absorption band near 260 nm almost disappeared. Hedgehog Cd_7-MT-I were more positive near 220 nm than Zn_7-MT-I, and Cd_7-MT-I had stronger positive absorption near 250 nm than Zn_7-MT-I had. Moreover, the strong negative absorption of Cd_7M-T-I was near 210 nm while that of Zn_7-MT-I was near 220 nm. In the CD spectra of the five isoforms, the stronger the positive absorption near 260 nm was, the weaker the negative absorption near 240 nm was, which was the typical features of CD spectra of Cd(II)-SH complex. The locations of positive and negative absorption band for all the five MT isoforms are consistent. The analysis of apo-MT spectra according to the method of Provencher and Glockner yielded a contribution of α-helix, β-sheet and unordered structure (Table 2). More than half of the amino acid residues emerged in unordered structure for apo-MTs. There are some irregular peaks between 200-220 nm in figure B, D and E. The reason is unclear.

Fig.5 showed that hedgehog MTs (MT-fI, MT-I, MT-fII and MT-II) had five raman bands near 680, 1320, 1440, 1670 and 2930 cm^{-1}. Apo-MTs had no evident raman band except near 2930 cm^{-1}. There is no clear difference of raman bands among the isoforms of hedgehog MTs.

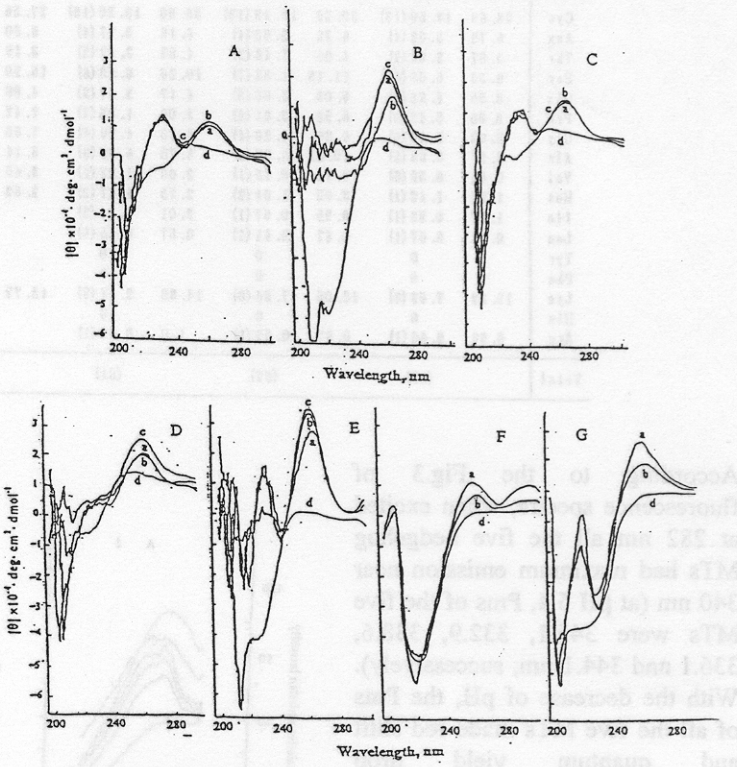

Fig. 4 Circular dichroism spectra of Cd-induced hedgehog MTs. The curves a, b, c, d relate to decreasing pH (5.4→3.0→2.0→1.0). Sample 2 ml, 50 mg/mL. A: MT-fI; B: MT-I; C: MT-fII; D: MT-II; E: MT-pII; F: Zn7-MT-I; G: Cd7-MT-I

Table 2 The Contribution of α-helix, β-sheet, β-turn and Unordered Structure of Hedgehog Apo-MTs

Structure	MT-fI	MT-I	MT-fII	MT-II	MT-pII
α-helix (%)	5	13	11	14	8
β-sheet (%)	17	9	10	7	16
β-turn (%)	21	18	20	15	18
Unordered structure(%)	57	60	59	64	58

DISCUSSIONS

The main structure characteristic of MT was its richness in cysteine, and MT chelates metal ions through -SH of cysteine. The hedgehog MTs are among the typical mammal MT. They have similar characteristics in UV, FS, CD and raman spectroscopy and take coincident change tendency of spectroscopic characteristic with pH decrease. These likeness indicated that they had similar mode of metal binding status and consequent three-dimension conformation.

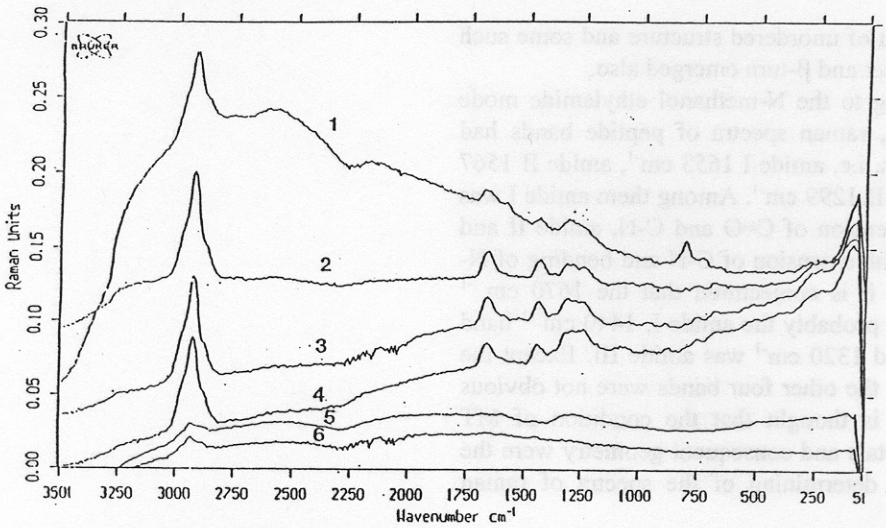

Fig. 5 Raman spectra of Cd-induced hedgehog MTs. 1: MT-fI; 2: MT-I; 3: MT-fII; 4: MT-II; 5: apo-MT-I; 6: apo-MT-II

1. Under comparatively high pH condition the Cd-induced hedgehog liver MTs had an obvious ultraviolet absorption shoulder near 250 nm (Fig.2), an evident fluorescent emission peak near 340 nm (Fig.3) and a strong positive CD band around 260 nm (Fig.4). They all came from the Cd(II)-SH chelating structure and the consequent compact conformation. When pH decreased all the 250 nm UV absorption shoulder, 340 nm FS peak and 260 nm CD positive absorption band diminished substantially, suggesting that the acid condition resulted in dissociation of metals from MT and loose structure. At low pH, the UV absorption shoulder near 250 nm disappeared and the 280 nm peak of disulfide bond did not appear. This indicated that at low pH the sulfydryl group of apo-MT was in the reduced state.

2. Generally said that the FS emitted by proteins came from the aromatic amino acid, especially, the fluorescence polarization of Tyr and Trp residues. MT from hedgehog have no aromatic amino acid (Table 1) and their fluorescent emission was from the metal-SH complex structure. The electric configuration of Cd^{+2} is $[Kr]4d^{8}5s^{2}$ and that of Zn^{+2} was $[Ar]3d^{8}4s^{2}$. The fact that the Pms appears comes from a relatively higher differential energy between s^{1} and s^{0} and a more compact state in the mini-environment of MT molecule. Following the lowering of pH in the solution, MT released metals step by step, and the quantum yield reduced and the structure of MT changed to loose structure with a red shift of fluorescence.

3. When pH was reduced, [θ] of the CD spectra of hedgehog MTs tended to decrease in the range of 200 to 300 nm, the positive absorption band tended to disappear and the negative concave became more negative. When pH decreased from 2.0 to 1.0, the absorption had an acute tending negative change. The sharp decrease of [θ] value formed a very negative peak near 210 nm or 230 nm, while the positive absorption near 260 nm disappeared. The reason was that the conformation of MT rapidly turned into a loose state as the pH decreased from 2.0 to 1.0 and metals were completely dissociated from MT following.

Under higher pH condition, the hedgehog MT-fI, MT-fII, MT-pII showed CD positive absorption band at 227, 231 and 232 nm successively, but the other two isoforms did not . The appearance of these positive band was perhaps the result of n-π* or π-π* transition in the amide group of the Cd(II)-SH chelate in MT peptide chain. No positive absorption at 230 nm may be the result of covery by negative absorption peak. Through the assay of Cd_7-MT-I and Zn_7-MT-I, it showed that the positive bands of Cd(II)-SH complex occurred at 226 nm and 250 nm, and that of Zn(II)-SH occurred at 245 nm. The former absorption band of 250 nm was located at longer wavelength than the latter one of 245 nm. The CD positive bands made red shift with the decrease of pH indicated that zinc was more easily released from the MT than cadmium.

With the lowering of pH, the five MTs from hedgehog showed different depth of the CD negative peak, which suggested that the conformation of the diverse isoforms was incompletely similar, and the state of metal binding of each isoform also varied to some extent. Because of no metal to stabilize the conformation and the partly folding of the peptide, the five apo-MT isoforms from hedgehog existed mainly in

the conformation of unordered structure and some such as α-helix, β-sheet and β-turn emerged also.

4. According to the N-methanol ethylamide mode experiment [10], raman spectra of peptide bands had three main peaks, i.e. amide I 1653 cm^{-1}, amide II 1567 cm^{-1} and amide III 1299 cm^{-1}. Among them amide I was the result of extension of C=O and C-N, amide II and amide III were the extension of C-N and bending of N-H on plane. So it is conjectured that the 1670 cm^{-1} raman band was probably the amide I, 1440 cm^{-1} band was amide II and 1320 cm^{-1} was amide III. Except the 2930 cm^{-1} band, the other four bands were not obvious for apo-MT. It is thought that the condition of MT binding with metals and consequent geometry were the major factor in determining of the spectra of raman scattering.

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