Cadmium-113 FT NMR-spectra of rabbit liver metallothioneins

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Summary. Cadmium-113 FT NMR-spectra of rabbit liver metallothionein-I and -II gave 6 and 7 NMR-signals, respectively, at between 610 and 670 ppm downfield from aqueous $Cd(ClO_4)_2$ solution (0.1 M).

Chemical and physicochemical analyses of heavy metals have been restricted to determining the total amount of metals by degradative methods such as atomic absorption analysis. Application of high resolution NMR-analysis to heavy metals, especially those with half nuclear spin, may give useful information and assist in understanding their chemical environments. So far such work has been largely concerned with ^{1}H, ^{13}C, ^{15}N and ^{31}P.

We report the results of high resolution FT NMR-experiments on cadmium-113 in cadmium-binding protein, metallothionein. Metallothionein (MT), which is a low mol. wt protein (6000–7000 daltons) and rich in both cysteinyl residues and metals (cadmium and zinc), was first isolated from equine kidney as a unique cadmium-binding protein[2]. Primary structures of equine renal MT-1B[3], mouse liver MT-I[4], and human hepatic MT-II[5] have been reported to contain 20 cysteinyl residues. The protein is inducible in the liver and kidneys of experimental animals by loading of zinc, cadmium, (possibly) copper and mercury[6].

Materials and methods. Rabbit liver MT were induced by repeated s.c. injections of ^{113}Cd-enriched (more than 90%) cadmium chloride to female rabbits (b.wt 2.5–2.8 kg; 0.5 mg Cd^{2+}/kg b.wt; 27 injections during 5 weeks). The animals were sacrificed 2 days after the last injection. The livers were homogenized in Tris buffer solution (0.1 M, pH 7.4) containing glucose (0.25 M) using a teflon homogenizer under nitrogen gas and the homogenate was centrifuged at 105,000 × g for 90 min at 2 °C. The supernatant

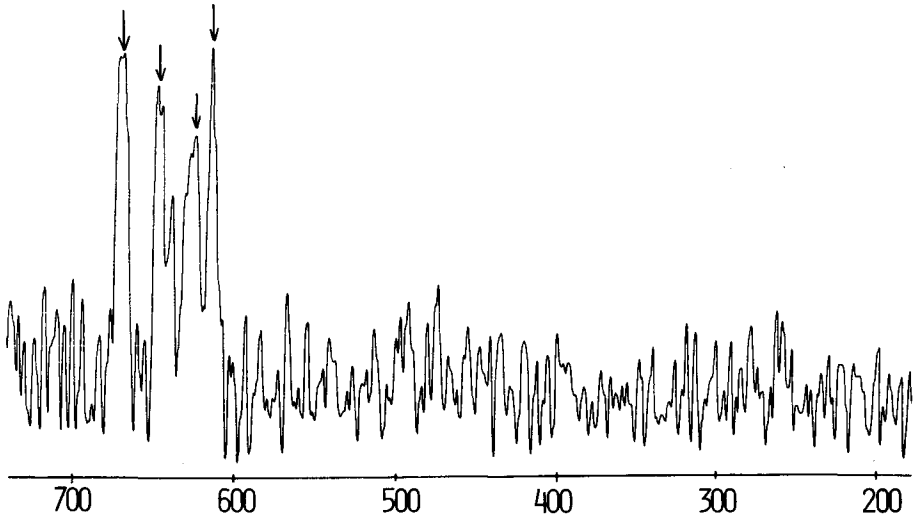

Fig. 1. Cd-113 FT NMR-spectrum of rabbit liver metallothionein-I. MT-I (2.48 mg Cd) in Tris buffer solution (1.2 ml, 10 mM, pH 8.6, 0.9% NaCl, 10% D_2O) was recorded on a JEOL FX-100 NMR-spectrometer with tunable probe at 22.02 MHz, spectral width 16 K, data points 4K/4K, 33,000 pulses (0.15 sec between pulses) and pulse width 25 μsec (90°). Ppm with respect to aqueous $Cd(ClO_4)_2$ solution (0.1 M), increasing values correspond to decreasing shielding.

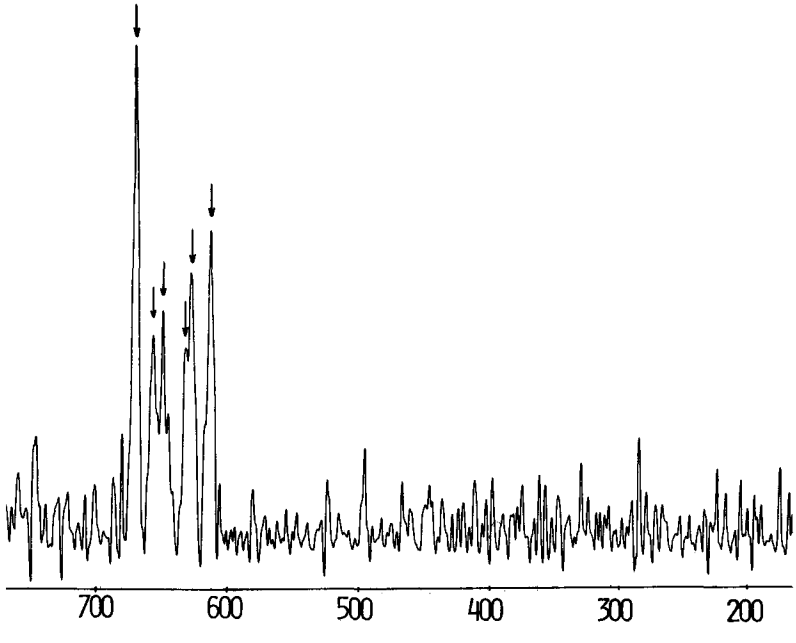

Fig. 2. Cd-113 FT NMR spectrum of rabbit liver metallothionein-II. MT-II (3.43 mg Cd) in Tris buffer solution (1.3 ml, 10 mM, pH 8.6, 0.9% NaCl, 10% D_2O) was recorded under the same conditions as MT-I.

(50 ml) was applied to a Sephadex G-75 column (5 × 80 cm) after the addition of $^{113}Cd^{2+}$ (1.5 mg) to replace zinc in MT and eluted with Tris buffer solution (10 mM, pH 8.6). The MT fractions (monitored by atomic absorption analysis of cadmium) were combined and concentrated by ultrafiltration on a Diaflo UM-10 membrane. The concentrated solution was then put in a DEAE Sephadex A-25 column (1.5 × 28 cm) to separate the 2 forms (MT-I and MT-II) by gradient elution with Tris buffer (pH 8.6) between 10 mM (100 ml) and 300 mM (400 ml). The separated MT-I and MT-II were concentrated to about 1 ml as above. The contents of cadmium, zinc, and copper in MT-I and MT-II were: 2.48, 7.9×10^{-3} and 44.8×10^{-3} mg/ml; 3,43, 27.2×10^{-3} and 10.7×10^{-3} mg/ml, respectively.

Results and discussion. Cadmium-113 high resolution NMR-spectra of each MT in 10 mm NMR-tube are shown in figures 1 and 2. Cadmium-113 peaks were observed between 610 and 670 ppm downfield from aqueous $Cd(ClO_4)_2$ solution (0.1 M). The chemical shifts were in accordance with the reported shifts of cadmium-sulfur complexes and indicated that cadmium in MT was coordinated with mercapto groups[7]. The number of signals can be summarized as follows: MT-I, 614.9 (1), 624.7 (2), 648.9 (1) and 671 (2); MT-II, 612.2 (1), 627.2 (1), 631.3 (1), 648.2 (1), 656.0 (1) and 669.6 ppm (2). Although the primary structure and the numbers of metals in rabbit liver MT are not known, the numbers of metals in the rabbit MT might be 7 as in the case of both forms of mouse liver MT[4]. 2 ^{113}Cd peaks may be present at 648 ppm (corresponding to 7 metals in MT-I) but broad signals prevented to clarify this. The similarity of both spectra indicated the close resemblance of amino acid sequences and chemical environments in MT-I and MT-II. Although the line width of inorganic cadmium is very narrow[8] (e.g., 0.49 Hz for 2 M $CdSO_4$), the broadening of line width was observed in the spectra of MT (more than 120 Hz) as observed in the case of Cd-alkaline phosphatase[9] and it severely reduced the high sensitivity of the nuclei. ^{13}C-enriched potassium cyanide was added to the MT solution and the ^{13}C NMR-spectra were recorded to observe separate ^{13}C signals with ^{13}C-^{113}Cd coupling. However, the spectra gave only a single ^{13}C peak which was shifted from a ^{13}C peak in the same solution without MT (unpublished observation). The result indicated that cadmium was strongly coordinated with sulfhydryl groups. 20 cysteinyl residues among the 61 amino acids in MT can be the primary coordinating groups to the metal, and it is considered that cadmium requires 1 (or more) coordinating group, including water, for the usual tetrahedral structure in MT.

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Experimental immunity against trypanosomiasis

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Summary. 9 groups of 6 female rats were used in an experiment using fraction 3 of *Trypanosoma rhodesiense*. 500 µg gave 100% immunoprotection and 1000 and 1500 µg gave 66% immunoprotection when challenged with 5×10^2 *T. brucei*. 2 groups of 10 female rats were tested for a short period inoculation immune response. In this, 750 µg of fraction 3 of *T. rhodesiense* gave 70% immunoprotection when challenged with *T. brucei*.

It has been demonstrated that subcellular particles or antigens of trypanosomes stimulate the formation of IgG antibodies, and that these particles display a common antigenicity across species[2-4]. Previous experiments have demonstrated higher IgG activity and greater immunoprotection from a centrifugal fraction termed fraction 3, when challenged with homologous and heterologous strains in mice and rats[4-7].

This report presents the results of 2 sets of experiments on inoculation with fraction 3 of *T. rhodesiense* in rats subsequently challenged with *T. brucei*. Fraction 3 of *T. rhodesiense* was isolated as previously described and alum was used continuously as adjuvant[7]. The rats were inoculated in the foot pads[7,8]. Blood was taken from the tail daily from day 3 after-challenge to day 30 to gage infection.

In the 1st experiment, 9 groups of 6 female rats of an average weight of 200 g were used. 1st group was kept as

Table 1.

Total dose of fraction 3 (µg)	Number of animals	Number with trypanosomes	Survival time (days) 6	12	Beyond 30
Control	6	6	1	0	0
0.1	6	6	1	0	0
1.0	6	6	4	0	0
33	6	6	2	0	0
100	6	6	6	6	0
500	6	0	6	6	6
1000	6	2	6	6	4
1500	6	2	6	6	4
2000	6	6	6	0	0

Table 2.

Experimental group	Number of animals	Number with trypanosomes	Survival time (days) 6	12	Beyond 30
Control	10	10	7	0	0
Fraction 3 Inoculated (750 µg)	10	3	10	10	7