Table 1. Isolation of *P. aeruginosa* with or without multidrug resistance in medical facilities

Isolates		2007	2008	2009
P. aeruginosa				
	Total numbers (No.) [a)]	228 449	233 301	223 232
	No. of isolates per 1 000 beds/year	723.0	738.4	706.5
TDR isolates	Total No.	14 340	14 043	13 009
	No. of isolates per 1 000 beds/year	45.4	44.4	41.2
	Ratio (%) [b)]	6.3	6.0	5.8
	Patient No. [c)]	6 789	6 474	6 231
	No. of patients per 1 000 beds/year	21.5	20.5	19.7
MDR isolates	Total No.	7 688	6 540	5 683
	No. of isolates per 1 000 beds/year	24.4	20.7	18.0
	Ratio (%)	3.4	2.8	2.5
	Patient No.	2 779	2 481	2 246
	No. of patients per 1 000 beds/year	8.8	7.9	7.1

[a)]: Numbers of *P. aeruginosa* isolated from the 771 medical facilities that responded to the questionnaire survey.

[b)]: Ratios of the numbers of TDR or MDR *P. aeruginosa* to the total numbers of *P. aeruginosa* isolates (%).

[c)]: Numbers of patients from whom TDR or MDR *P. aeruginosa* were isolated.

Table 2. Isolation of *Acinetobacter* spp.. with or without multidrug resistance in medical facilities

Isolates		2007	2008	2009
Acinetobacter spp..				
	Total numbers (No.) [a]	32 073	31 330	30 609
	No. of isolates per 1 000 beds/year	101.5	99.2	96.9
	Patient No. [b]	20 782	20 394	19 625
	No. of patients per 1 000 beds/year	65.8	64.5	62.1
MDR isolates				
	Total No.	84	143	331
	No. of isolates per 1 000 beds/year	0.3	0.5	1.1
	Rate (%)[c]	0.3	0.5	1.1
	Patient No.	51	81	97
	No. of patients per 1 000 beds/year	0.2	0.3	0.3

[a]: Numbers of *Acinetobacter* spp.. isolated from the 771 medical facilities that responded to the questionnaire survey.

[b]: Numbers of patients from whom MDR *Acinetobacter* spp.. were isolated.

[c]: Ratio of numbers of MDR *Acinetobacter* spp.. to the total numbers of *Acinetobacter* spp.. isolated (%)

444 Table 3 Isolation of *Acinetobacter* spp.. With or without multidrug resistance in medical facilities by spp.

Isolates		2007	2008	2009
(No. of medical facilities where species-level identification was performed)		(558)	(571)	(577)
A. baumannii	Total numbers (No.) [a)]	21 116	20 371	20 307
	No. of isolates per 1 000 beds/year	87.1	82.1	81.2
A. lwoffii	Total No.	3 011	3 134	2 838
	No. of isolates per 1 000 beds/year	12.4	12.6	11.4
A. calcoaceticus	Total No.	1 124	1 059	1 144
	No. of isolates per 1 000 beds/year	4.6	4.3	4.6
Other *Acinetobacter* spp..	Total No.	4 125	4 469	4 136
	No. of isolates per 1 000 beds/year	17.0	18.0	16.5
(No. of medical facilities where MDR strains were isolated)		(34)	(33)	(45)
MDR *A. baumannii*	Total No.	35	96	292
	No. of isolates per 1 000 beds/year	0.1	0.4	1.2
	Ratio (%) [b)]	0.2	0.5	1.4
MDR *A. lwoffii*	Total No.	21	12	6
	No. of isolates per 1 000 beds/year	0.1	0.05	0.02
	Ratio (%)	0.7	0.4	0.2
MDR *A. calcoaceticus*	Total No.	3	1	0
	No. of isolates per 1 000 beds/year	0.01	0.004	0
	Ratio (%)	0.3	0.1	0
Other MDR spp..	Total No.	11	18	20
	No. of isolates per 1 000 beds/year	0.05	0.07	0.08
	Ratio (%)	0.3	0.4	0.5

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446 a): Numbers of *Acinetobacter* spp.. isolated from the 771 medical facilities that responded to the questionnaire survey.

447 b): Ratio of the numbers of MDR *Acinetobacter* spp.. to the total numbers of *Acinetobacter* spp.. isolated (%).

448

Figure 1

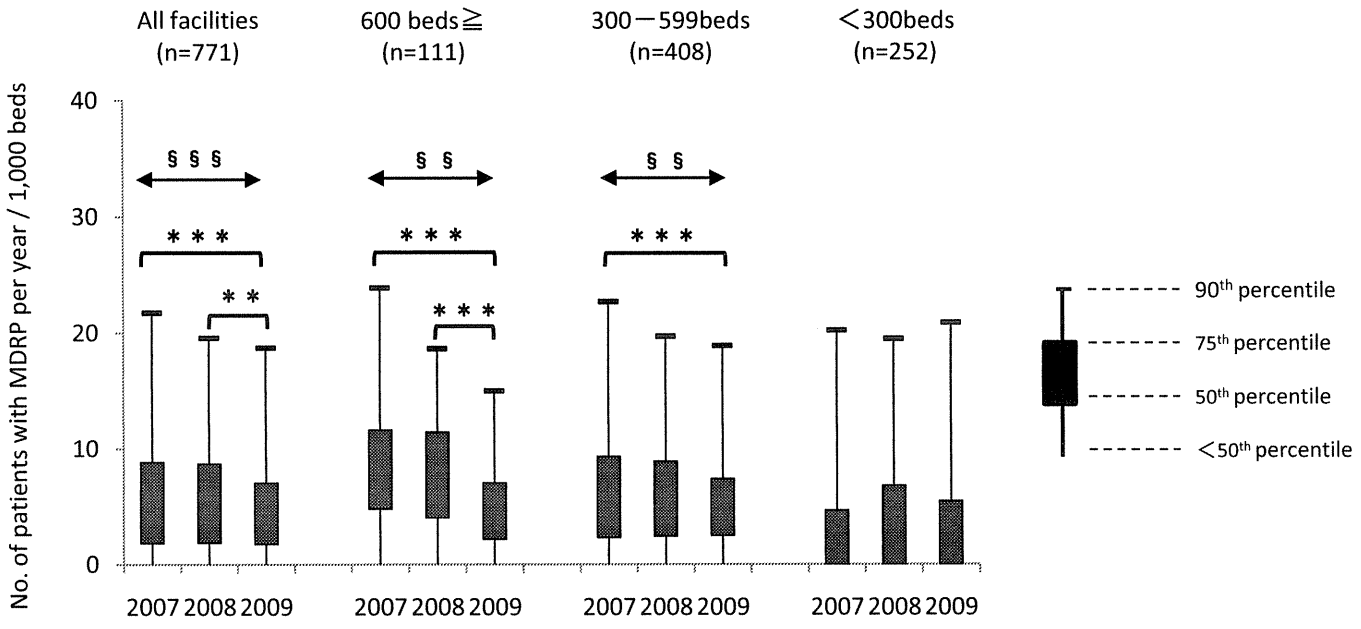

All facilities
(n=771)
600 beds≧
(n=111)
300－599beds
(n=408)
＜300beds
(n=252)
No. of patients with MDRP per year / 1,000 beds
40
30
20
10
0
§ § §
§ §
§ §
* * *
* * *
* * *
* *
* * *
2007 2008 2009
2007 2008 2009
2007 2008 2009
2007 2008 2009
90th percentile
75th percentile
50th percentile
＜50th percentile

Figure 2

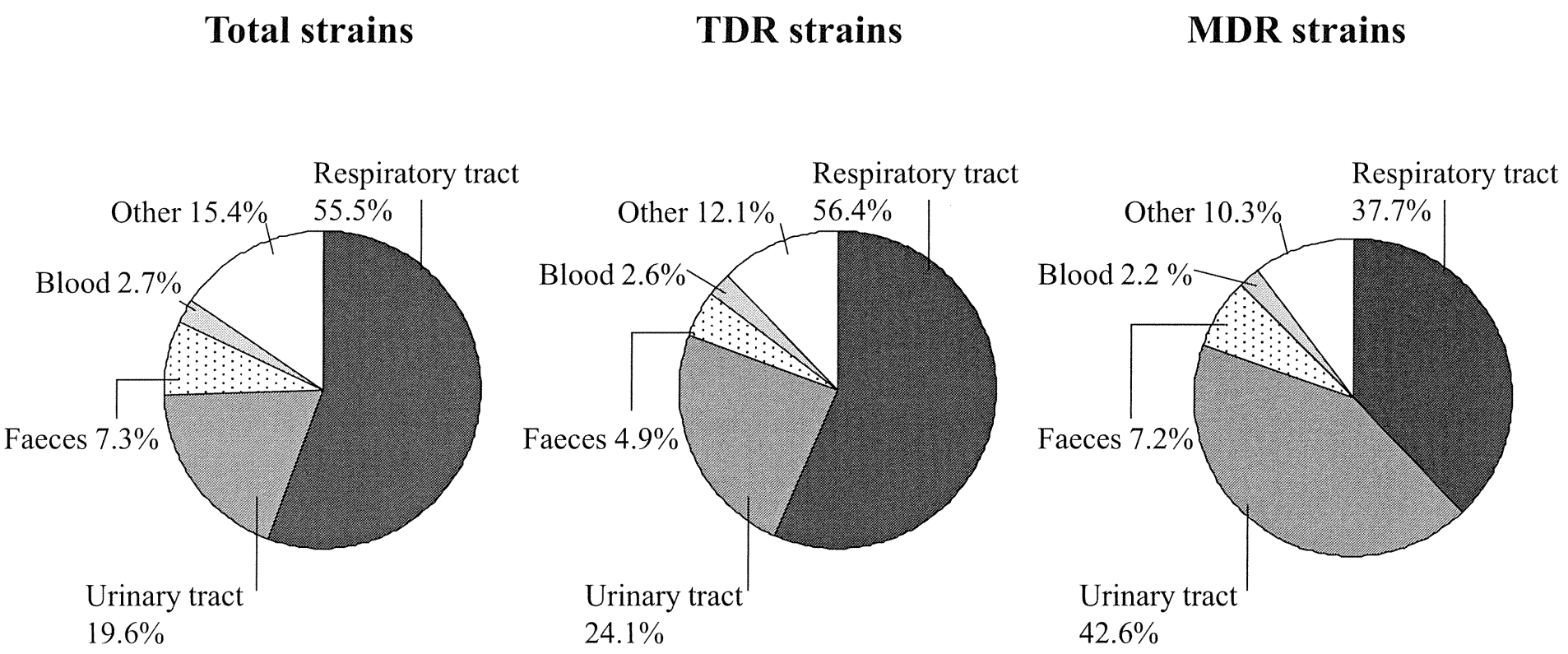
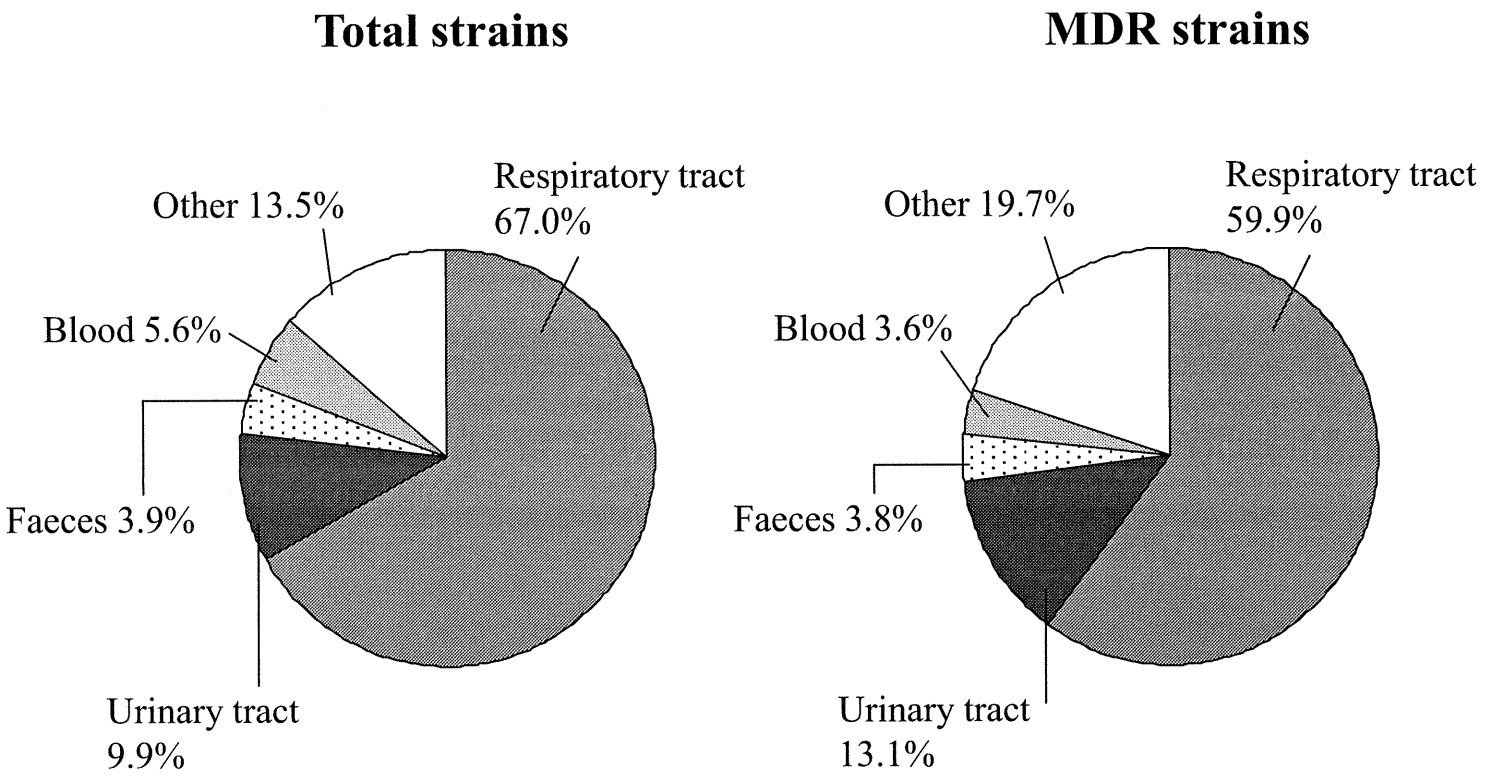

Total strains
Respiratory tract 55.5%
Other 15.4%
Blood 2.7%
Faeces 7.3%
Urinary tract 19.6%
TDR strains
Respiratory tract 56.4%
Other 12.1%
Blood 2.6%
Faeces 4.9%
Urinary tract 24.1%
MDR strains
Respiratory tract 37.7%
Other 10.3%
Blood 2.2 %
Faeces 7.2%
Urinary tract 42.6%

Figure 3


Total strains
Other 13.5%
Respiratory tract
67.0%
Blood 5.6%
Faeces 3.9%
Urinary tract
9.9%
MDR strains
Other 19.7%
Respiratory tract
59.9%
Blood 3.6%
Faeces 3.8%
Urinary tract
13.1%

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CONCISE ARTICLE

Metal preference of Zn(II) and Co(II) for the dinuclear metal binding site of IMP-1 metallo-β-lactamase and spectroscopic properties of Co(II)-substituted IMP-1 with mercaptoacetic acid†

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IMP-1 metallo-β-lactamase is a dinuclear Zn(II) enzyme that catalyzes the hydrolysis and inactivation of most β-lactams including carbapenems, and is involved in one of the mechanisms for generating clinical resistance to antibiotics in pathogenic bacteria. We investigated the metal preferences of Zn(II) and Co(II) for the apo-enzyme of IMP-1 metallo-β-lactamase, apo-IMP-1, which contains a dinuclear metal binding site (the Zn1 and Zn2 sites), by UV-visible spectroscopy. The UV-visible spectrum of apo-IMP-1 containing 1 equiv. of Co(II) and 1 equiv. of Zn(II) showed a high preference of Zn(II) for the Zn1 site compared to Co(II). Moreover, Zn(II) bound more strongly to the Zn2 site than Co(II). The interaction of IMP-1 metallo-β-lactamase with mercaptoacetic acid was also investigated using Co(II)-substituted IMP-1 and UV-visible spectroscopy. Possible metal binding modes of Co(II) or Zn(II) to the dinuclear metal binding site in apo-IMP-1 and of mercaptoacetic acid to Co(II)-substituted IMP-1 are proposed.

Introduction

Metallo-β-lactamases are Zn(II)-dependent enzymes that catalyze the hydrolysis of most β-lactams including carbapenems with the exception of monobactams such as aztreonam, and are now recognized as a new potential threat to human society. Some metallo-β-lactamases including IMP-1 discovered in Japan are coded by a gene cassette in an integron and are transferable to other bacteria.[1,2] Since a new metallo-β-lactamase, NDM-1, has just emerged in India and Pakistan, and also been expanding in the United Kingdom[3] and in other countries, further worldwide proliferation of the kind of metallo-β-lactamases is becoming a grave general concern.[4] One countermeasure to these lactamases involves the development of inhibitors that block the action of the enzyme. At present, no relevant inhibitors are available for clinical use. In our continuing efforts to develop metallo-β-lactamase inhibitors, we found that mercaptocarboxylic acids are potent inhibitors for IMP-1.[5] Among them, mercaptoacetic acid and 2-mercaptopropionic acid strongly and competitively inhibited IMP-1 with K_i values of 0.23 and 0.19 μM, respectively.[5]

Based on the inhibition effect of mercaptoacetic acid against IMP-1, Arakawa *et al.* developed a double-disk method for detection of metallo-β-lactamase-producing Gram-negative bacteria.[6] This disk (named SMA disk) is composed of two Kirby–Bauer disks containing ceftazidime and a filter disk containing sodium mercaptoacetate. By changing the shape of the growth inhibitory zone around the disk containing ceftazidime or imipenem, the presence or absence of metallo-β-lactamase-producing bacteria could be judged.

In 2000, the X-ray crystal structure of IMP-1 was determined (Fig. 1).[7] IMP-1 is composed of an αβ/βα fold and two distinct Zn(II) binding sites, which are located at the bottom of a wide shallow groove between the β-sheets. One of the Zn(II) ions, Zn1, is tetrahedrally coordinated by three histidine residues (His116, His118, and His196, according to BBL numbering scheme[8]). Another Zn(II) ion, Zn2, is trigonal-bipyramidally coordinated by Asp120, Cys221, His263, and a water molecule. In addition, the water molecule is bridged to the two Zn(II) ions (presumably,

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† Electronic supplementary information (ESI) available: UV-visible spectral changes of Co(II) with varying concentrations of mercaptoacetic acid (Fig. S1). See DOI: 10.1039/c1md00062d

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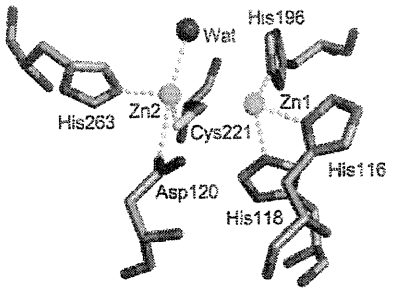

Fig. 1 Schematic representation of the active site of IMP-1 from *Pseudomonas aeruginosa* (PDB code 1DDK). The Zn(II) atoms and a water molecule are shown as spheres.

as a hydroxide ion), although the electron density of this water molecule was not observed in the X-ray crystal structure.

The relationship between the role of each metal ion in the active site and its catalytic activity in metallo-β-lactamases remains controversial. Unfortunately, spectroscopic studies to characterize the metal binding sites have been limited, due to the physicochemical properties of Zn(II). It would be beneficial to exchange Zn(II) with Co(II), which yields a structurally similar and catalytically active analog. One method to prepare Co(II)-substituted enzymes of metallo-β-lactamases is the direct addition of Co(II) to an apo-enzyme that is prepared by the use of chelators and dialysis. The preparation of apo- and Co(II)-substituted enzymes of BcII and CcrA metallo-β-lactamases has been successful.[9–13] Although the preparation of the apo-enzyme of IMP-1 metallo-β-lactamase, apo-IMP-1, has been attempted, complete demetallation with chelators such as EDTA resulted in failure. Very recently, we established a preparation technique for apo-IMP-1 metallo-β-lactamase, which has restored enzyme activity upon addition of Zn(II) ions.[14] Using this technique, we were able to investigate spectroscopic studies on Co(II)-substituted IMP-1.

In this paper, we investigated the metal preferences of Co(II) or Zn(II) for apo-IMP-1 and the spectral properties of mercaptoacetic acid with Co(II)-substituted IMP-1, using UV-visible spectroscopy.

Results and discussion

Metallo-β-lactamases are Zn(II)-dependent enzymes that hydrolyze most β-lactams, even in environments in which the Zn(II) concentration is extremely low. Analysis of the coordination environment around the Zn(II) active site is of great importance for elucidating the relationship between the role of individual Zn (II) ions and the catalytic mechanism of metallo-β-lactamases. Zn (II) ions are, however, "spectroscopically silent" d^{10} metal ions. Therefore, substitution of Zn(II) by Co(II) has become an essential technique to address the structural basis of catalytic properties in Zn(II) enzymes.[15] From this standpoint, the preparation of an apo-enzyme is a crucial step.

For metallo-β-lactamases, apo-enzymes of BcII and CcrA have been prepared and spectroscopic properties of their Co(II)-substituted enzymes were investigated.[9–13] In IMP-1, however, apo-enzymes prepared by the dialysis method were not recovered perfectly after the addition of excess $Zn(NO_3)_2$,[16,17] and the quest for the preparation of an apo-enzyme and metal substitution is ongoing.

Recently, we established a preparation method for apo-IMP-1 with a combination of the chelating agent, EDTA, and a desalting technique with a PD-10 column.[14] As a result, upon addition of Zn(II) ions to the prepared apo-IMP-1, the enzyme activity could be recovered comparable to that observed with the native enzyme. Thus, this preparation allowed us to investigate the spectroscopic properties of Co(II)-substituted IMP-1.

Binding of Co(II) to apo-IMP-1

We previously carried out the spectrophotometric titration of apo-IMP-1 with Co(II) in 50 mM MOPS–NaOH (pH 7.0), 1.0 M NaCl, and 30% glycerol.[14] The UV-visible spectral changes in 50 mM Tris–HCl (pH 7.4), 1.0 M NaCl, and 25% glycerol are shown in Fig. 2A. Note that the spectra were almost identical even when taken in the different buffer solutions. Successive addition of Co(II) to apo-IMP-1 exhibited an intense S^- of Cys221 → Co(II) ligand-to-metal charge transfer (LMCT) band at 350 nm and d–d absorptions in the range of 500–650 nm. The assignment of the LMCT band at 350 nm is supported by disappearance of this band in the UV-visible spectra of the Co(II)-substituted mutants of IMP-1 and other metallo-β-lactamases,[9,11,14] where the Cys221 ligand is replaced by Ala or Ser. Absorbances in both the LMCT and d–d bands continued to increase as Co(II) was added, reaching a plateau at 2 equiv. per apo-IMP-1. The spectrum of apo-IMP-1 containing 1.0 equiv. of Co(II) exhibited absorption maxima at 350, 520, 552, 612, and 635 nm (Fig. 2A). Judging from the increase in the absorbance of the LMCT band at 350 nm, Co(II) distributes between the two sites in the ratio of 2 in the Zn1 site to 1 in the Zn2 site. This result agrees with that for BcII reported by de Seny *et al.*[11] Macroscopic dissociation constants for apo-IMP-1 with Co(II) were derived from spectral changes of the enzyme upon Co(II) binding, using the program DynaFit,[18] and found to be $K_{D1} < 60$ nM and $K_{D2} = 0.3$ μM.[14]

Binding selectivity of Co(II) and Zn(II) to the Zn1 and Zn2 sites in apo-IMP-1

Next, we investigated the preference of Co(II) and Zn(II) for two metal binding sites. Spectral changes of apo-IMP-1 upon successive addition of 1 equiv. of Co(II), followed by the addition of Zn(II) (by 0.2 equiv. increments for each metal) in 50 mM Tris–HCl (pH 7.4), 1.0 M NaCl, and 25% glycerol are shown in Fig. 2B. As described above, the spectrum of apo-IMP-1 containing 1 equiv. of Co(II) exhibited absorption maxima at 350, 520, 552, 612, and 635 nm. Addition of 1 equiv. of Zn(II) to apo-IMP-1 containing 1 equiv. of Co(II) resulted in the increase in the absorbance of the LMCT band at 350 nm, whereas those of the d–d bands in the visible range did not increase significantly with 1 equiv. of Zn(II) added. The increase in the LMCT band at 350 nm is indicative of Co(II) binding at the Zn2 site.[9,11,13,14,19,20] Therefore, this spectral behaviour seems to indicate that Co(II) is pushed out from the Zn1 site with an occupation of Zn(II) at the Zn1 site and shifted to the Zn2 site. This result suggests that Zn (II) preferentially binds to the Zn1 site over the Zn2 site.

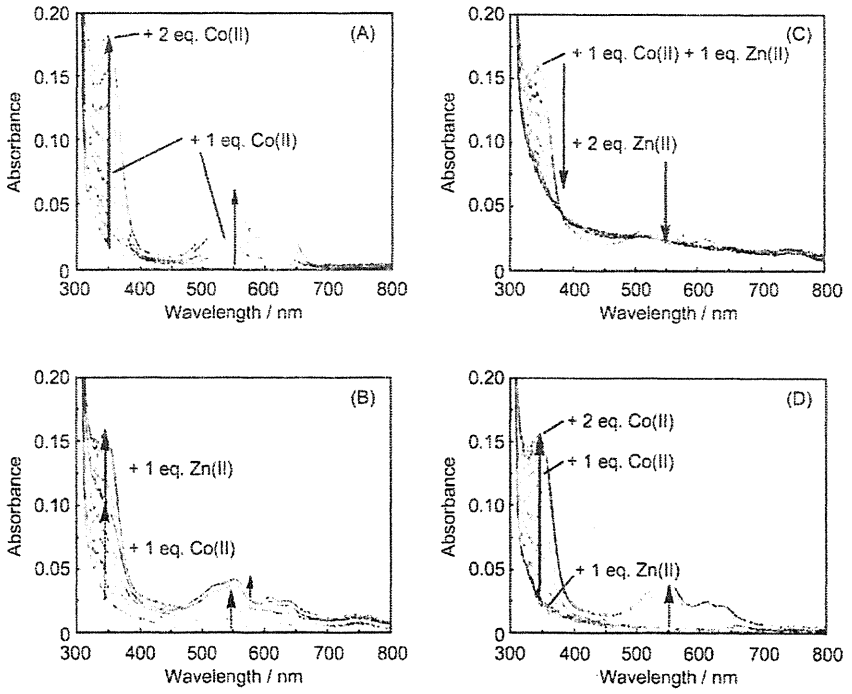

Fig. 2 UV-visible spectral changes of apo-IMP-1 with varying concentrations of Co(II) or Zn(II). (A) Solid arrow: apo-IMP-1 (150 μM) in 50 mM Tris–HCl (pH 7.4), 1.0 M NaCl and 25% glycerol was titrated by 0.2 equiv. of Co(II) to a total of 2 equiv. (B) 1 equiv. of Co(II) in increments of 0.2 equiv. was added to apo-IMP-1 (178 μM) in 50 mM Tris–HCl (pH 7.4), 1.0 M NaCl, and 25% glycerol (dashed arrow), followed by the addition of Zn(II) (0.2 equiv. increments) up to 1 equiv. (solid arrow). (C) 2 equiv. of Zn(II) in increments of 0.2 equiv. was added to apo-IMP-1 containing 1 equiv. of Co(II) and 1 equiv. of Zn(II) (see final spectrum in (B)). (D) 1 equiv. of Zn(II) in increments of 0.2 equiv. was added to apo-IMP-1 (178 μM) in 50 mM Tris–HCl (pH 7.4), 1.0 M NaCl, and 25% glycerol, followed by the addition of Co(II) (0.2 equiv. increment) up to 2 equiv. (solid arrow).

When 2 equiv. of Zn(II) was added to apo-IMP-1 containing 1 equiv. of Co(II) and 1 equiv. of Zn(II), the absorption bands at 350 nm and 500–650 nm disappeared as the concentration of Zn (II) increased (Fig. 2C), suggesting that the replacement of Co(II) by Zn(II) takes place at both the Zn1 and the Zn2 sites, suggesting a higher preference of Zn(II) for both the Zn2 and Zn1 sites than Co(II).

Fig. 2D shows spectral changes of apo-IMP-1 upon successive addition of 1 equiv. of Zn(II), followed by the addition of 2 equiv. of Co(II) (by 0.2 equiv. increment for each metal) in 50 mM Tris–HCl (pH 7.4), 1.0 M NaCl, and 25% glycerol. No change in the spectrum of apo-IMP-1 was observed in the presence of 1 equiv. of Zn(II). The addition of Co(II) resulted in the increase in both the LMCT and d–d bands, where the final spectrum is consistent with that in Fig. 2B, suggesting that Co(II) binds to the Zn2 site. Interestingly, the extinction coefficients (ε) of the LMCT band at 350 nm are *ca.* 730 and *ca.* 900 M^{-1} cm^{-1}, respectively, when 1 and 2 equiv. of Co(II) were added after addition of 1 equiv. of Zn(II) to apo-IMP-1. These values are smaller than that of apo-IMP-1 in the presence of 2 equiv. of Co(II) [Fig. 2A, ε = *ca.* 1200 M^{-1} cm^{-1}, that is, Co(II) totally occupies the Zn2 site], suggesting that Zn(II) was already distributed between the Zn1 and Zn2 sites before addition of Co(II).

Preliminary differential scanning calorimetry measurements also showed enhancement in the denaturation temperature (T_d) from 56.4 °C for apo-IMP-1 or 56.5 °C for mono-Zn(II)-IMP-1 [apo-IMP-1 plus 1 equiv. of Zn(II)] to 76.9 °C for native IMP-1.[14] These analyses revealed that Zn1 contributes to the enzyme activity, whereas Zn2 plays an important role both in stabilizing the protein structure and in increasing catalytic efficiency of the enzyme.

Interaction of mercaptoacetic acid with Co(II)-substituted IMP-1

The coordination of the thiolate group in mercaptoacetic acid to a metal ion in the active site was followed by UV-visible spectroscopy of Co(II)-substituted IMP-1 prepared from apo-IMP-1 and 2 equiv. of Co(II) with mercaptoacetic acid. The spectral changes of Co(II)-substituted IMP-1 with mercaptoacetic acid in 50 mM Tris–HCl (pH 7.4), 1.0 M NaCl, and 25% glycerol are shown in Fig. 3. The addition of mercaptoacetic acid to Co(II)-substituted IMP-1 up to 2 equiv. resulted in a marked increase in the absorbance of the S^--to-Co(II) LMCT band at 350 nm and a shift of the d–d bands (555, 597, and 635 nm) with an increase in the absorbance (Fig. 3). The former feature is characteristic of the S^- of mercaptoacetic acid → Co(II) LMCT band, implicating coordination of the thiolate group of mercaptoacetic acid to Co (II). Bicknell *et al.* prepared Co(II)-substituted angiotensin converting enzyme [Co(II)-ACE] and characterized the catalytic metal binding site both in Co(II)-ACE and in its inhibitor, captopril, by UV-visible spectroscopy.[21] The visible spectrum of Co(II)-ACE exhibits a single broad maximum at 525 nm (ε = 75 M^{-1} cm^{-1}).[21] Upon addition of captopril to Co(II)-ACE, the spectrum of Co(II)-ACE–captopril complex displays peaks at 540 (ε = 350 M^{-1} cm^{-1}), 618 (520), and 637 (560) nm, indicating that maxima at longer wavelength and the increase in the absorbance are changes of the d–d transition of Co(II) by inhibitor binding to the active-site metal. In IMP-1, the spectra of

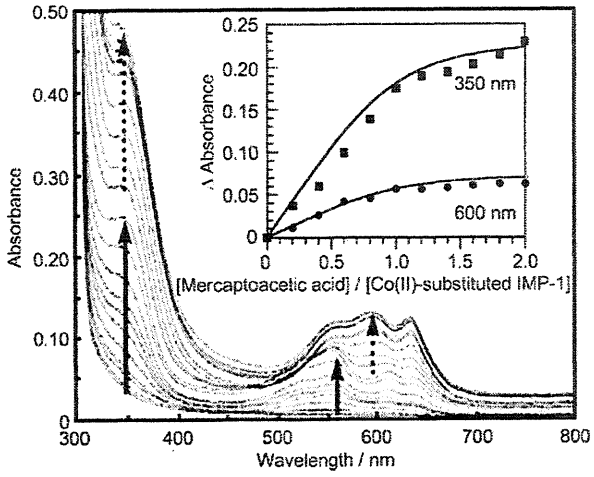

Fig. 3 Spectrophotometric titration of Co(II)-substituted IMP-1 with mercaptoacetic acid. Solid arrow: apo-IMP-1 (171 μM) in 50 mM Tris–HCl (pH 7.4), 1.0 M NaCl, and 25% glycerol was titrated by 0.2 equiv. of Co(II) to a total of 2 equiv. Dashed arrow: 2 equiv. of mercaptoacetic acid in increments of 0.2 equiv. was added to Co(II)-substituted IMP-1 [apo-IMP-1 containing 2 equiv. of Co(II)]. Inset: plot of the absorbances at 350 nm (squares) and 600 nm (circles) as a function of the concentration of added mercaptoacetic acid. The absorbances of apo-IMP-1 and Co(II)-substituted IMP-1 were subtracted from the absorbance of Co(II)-substituted IMP-1 added mercaptoacetic acid. The solid lines represent fits obtained from numerical simulation of a one-step binding model to the data using the program Dynafit (BioKin, Ltd.).[18] The apparent dissociation constant obtained was 14 μM.

Co(II)-substituted IMP-1–mercaptoacetic acid have two features: (i) enhanced absorbance without change in wavelength and (ii) a change in absorption that appears to be a new absorption rather than a shift in an absorption band. This spectral change is similar to that of Co(II)-BcII–D-captopril complex.[22]

When changes in the absorbances at 350 and 650 nm were plotted as a function of the concentration of added mercaptoacetic acid, a plateau was observed for the [mercaptoacetic acid]/[Co(II)-substituted IMP-1] ratio above 1 (Fig. 3, inset), indicating that mercaptoacetic acid binds to Co(II)-substituted IMP-1 to form a 1 : 1 complex. The apparent dissociation constant of mercaptoacetic acid with Co(II)-substituted IMP-1 was estimated by nonlinear least-squares fitting of the spectrophotometric titration data using the program DynaFit,[18] and found to be K_D = 14 μM. In control experiments, it could be ruled out that mercaptoacetic acid binds to a free Co(II) ion because the spectra of $Co(II)SO_4$ in 50 mM Tris–HCl (pH 7.4), 1.0 M NaCl, and 25% glycerol are quite different from those of Co(II)-IMP-1–mercaptoacetic acid complex when mercaptoacetic acid is added from 0 to 2 equiv. (Fig. S1, ESI†).

Antony *et al.* applied the polarizable molecular mechanics method SIBFA to search for the most stable binding modes of captopril and thiomandelate inhibitors to a 104-residue model of CcrA metallo-β-lactamase.[23,24] The most stably bound complex is a monodentate complex, in which S^- bridges the two Zn(II) ions, with the carboxylate and carbonyl groups in captopril or the carboxylate group in thiomandelate interacting with the nearest residues around the dinuclear metal binding site. These

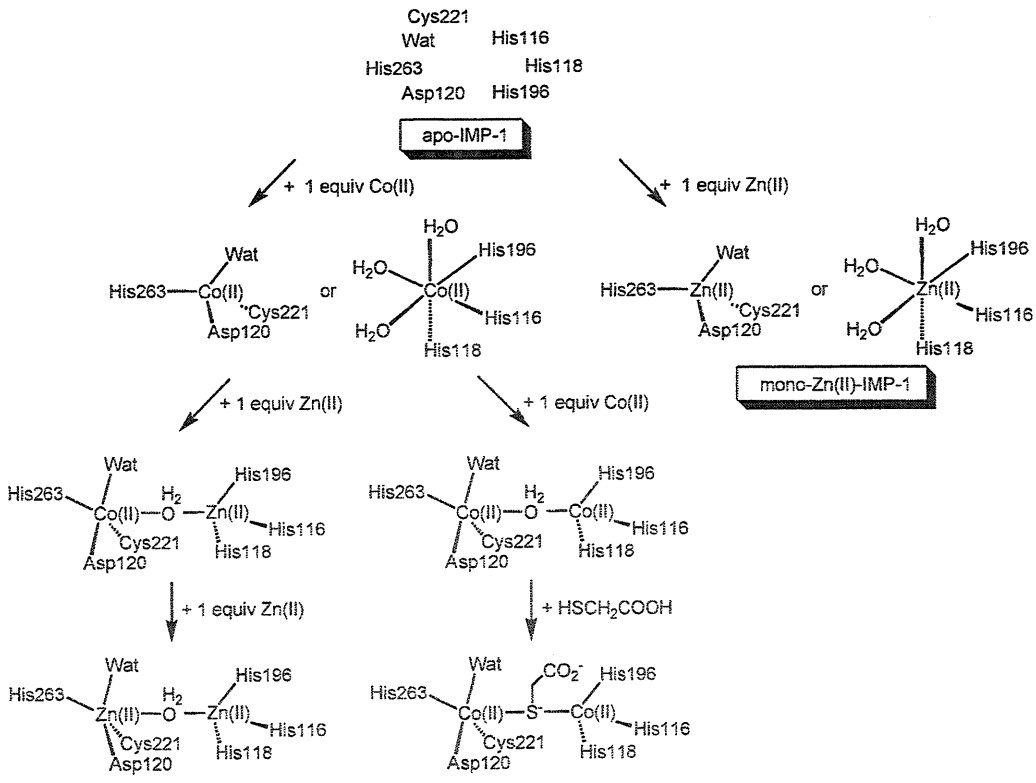

Fig. 4 Proposed modes of reconstruction of IMP-1 from apo-IMP-1 by Zn(II) and Co(II) and the interaction of Co(II)-substituted IMP-1 with mercaptoacetic acid.

are consistent with the X-ray crystal structures of BlaB metallo-β-lactamase complexed with D-captopril[25] and IMP-1 or VIM-2 metallo-β-lactamase complexed with mercaptocarboxylate inhibitors.[7,26]

Considering the results of UV-visible spectroscopy of Co(II)-substituted IMP-1 with mercaptoacetic acid, molecular mechanics calculations, and X-ray crystallography, it was concluded that the thiolate group in mercaptoacetic acid bridges the two Co(II) ions in the active site (Fig. 4).

Conclusions

In summary, we have investigated the metal preference of Co(II) and Zn(II) for dinuclear metal binding sites in apo-IMP-1 and the interaction of Co(II)-substituted IMP-1 with mercaptoacetic acid. We proposed models for the reconstruction of IMP-1 from apo-IMP-1 by Zn(II) and Co(II) and for the interaction of Co(II)-substituted IMP-1 with mercaptoacetic acid (Fig. 4). One equiv. of Co(II) or Zn(II) distributes between both the Zn1 and Zn2 sites. Metal binding modes for Co(II) and Zn(II) to apo-IMP-1 are similar to those found in BcII metallo-β-lactamase.[11,13] Unlike IMP-1 and BcII metallo-β-lactamases, CcrA and Bla2 metallo-β-lactamases showed that Zn(II) binding to apo-enzyme was sequential.[12,19]

Based on the results of UV-visible spectroscopy of Co(II)-substituted IMP-1 with mercaptoacetic acid, the inhibitory effect of mercaptoacetic acid using the double-disk method for detection of metallo-β-lactamase-producing Gram-negative bacteria is thought to arise from the coordination of the thiolate group in mercaptoacetic acid to two Zn(II) ions present in the active site of the metallo-β-lactamase.

Experimental

Chemicals

Antibiotics, cephaloridine from Shionogi & Co., Ltd. (Osaka, Japan) and ampicillin from Meiji Seika Co., Ltd. (Tokyo, Japan), were kindly donated. Cephalothin was purchased from Sigma-Aldrich Co., Ltd. (St Louis, USA). Disodium ethylenediaminetetraacetate (Na_2EDTA), glycerol, and tris(hydroxymethyl)aminomethane (Tris) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Mercaptoacetic acid was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). $CoSO_4 \cdot 7H_2O$, ultrapure water, and 3-morpholinopropanesulfonic acid (MOPS) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). $Zn(NO_3)_2 \cdot 6H_2O$ was purchased from Katayama Chemical Industries Co., Ltd. (Osaka, Japan). The other reagents were obtained commercially and were of the highest quality available.

Preparation of enzyme

IMP-1 metallo-β-lactamase from *Serratia marcescens* was prepared from the extracts of *Escherichia coli* JM109, which harbored plasmid pSMBNU24 containing the gene, bla_{IMP-1}, of *S. marcescens* TN9106, according to the published method.[5,27] *E. coli* JM109 cells were cultured in the Luria–Bertani medium containing 50 μg mL^{-1} ampicillin at 37 °C for 13 h. The cells were collected by centrifugation (10 000 × *g*) at 4 °C for 15 min and resuspended in 50 mM phosphate buffer (pH 7.0) containing 2 μM $Zn(NO_3)_2$. A crude enzyme solution was obtained from the suspension by sonication followed by centrifugation (150 000 × *g*) at 4 °C for 1 h. IMP-1 was purified by a SP Sepharose Fast Flow column (ϕ 26 mm × 10 cm; flow rate, 60 mL h^{-1}; GE Healthcare UK Ltd., UK) using an eluent of 50 mM phosphate buffer containing 2 μM $Zn(NO_3)_2$ with a gradient of 0 to 0.4 M NaCl in 10 mL fractions and a Sephadex G-75 (ϕ 16 mm × 90 cm; flow rate, 12 mL h^{-1}; GE Healthcare UK Ltd, Buckinghamshire, UK) with 50 mM Tris–HCl buffer (pH 7.4) containing 2 μM Zn $(NO_3)_2$. The fractions (3 mL each) that showed enzymatic activity were collected and concentrated by ultrafiltration (YM-10, Millipore Co., MA, USA). The purity of the enzyme was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; PAGEL, SPU-15S, ATTO, Tokyo, Japan) by comparison with protein molecular markers (Oriental Industries Co., Ltd., Tokyo, Japan). The concentration of the purified IMP-1 enzyme was determined by measuring the absorbance at 280 nm of enzyme preparation using an extinction coefficient of 4.9×10^4 M^{-1} cm^{-1}.

β-Lactamase activity

Unless stated otherwise, a spectrophotometric method was used to measure the initial rate of consumption of a substrate, cephalothin or cephaloridine, by the enzyme.

Preparation of apo-IMP-1

Apo-IMP-1 was prepared by a combination of EDTA and desalting column chromatography (PD-10, GE Healthcare UK Ltd., UK) according to our previously reported procedure.[14]

UV-visible spectroscopy. Apo-IMP-1 (150–178 μM) in 50 mM Tris–HCl (pH 7.4), 1.0 M NaCl, and 25% glycerol was titrated by the successive addition of 0.2 equiv. of a $Zn(NO_3)_2$ or $CoSO_4$ stock solution prepared in 50 mM Tris–HCl (pH 7.4), 1.0 M NaCl, and 25% glycerol.

In the spectrophotometric titration of Co(II)-substituted IMP-1 with mercaptoacetic acid, Apo-IMP-1 (171 μM) in 50 mM Tris–HCl (pH 7.4), 1.0 M NaCl, and 25% glycerol was titrated by the sequential addition of 0.2 equiv. of a mercaptoacetic acid stock solution prepared in 50 mM Tris–HCl (pH 7.4), 1.0 M NaCl, and 25% glycerol. UV-visible spectra were recorded at room temperature on a Shimadzu UV-2200 spectrophotometer (Kyoto, Japan), using 1 cm microcells. The interval of each titration was 5 min.

The apparent dissociation constant for mercaptoacetic acid with Co(II)-substituted IMP-1 was derived from spectral changes in absorbances at 350 and 650 nm with increasing concentration of mercaptoacetic acid, using the program DynaFit (BioKin, Ltd.).[18]

The following equilibrium was used for fitting.

$$\text{Co(II)-substituted IMP-1} + \text{mercaptoacetic acid} \overset{K_D}{\rightleftharpoons} \text{Co(II)-substituted IMP-1-mercaptoacetic acid}$$

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A Demetallation Method for IMP-1 Metallo-β-Lactamase with Restored Enzymatic Activity Upon Addition of Metal Ion(s)

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Metallo-β-lactamases (MBLs) are Zn^{II}-dependent enzymes that hydrolyze most β-lactams[1] and pose a potential threat in clinical environments due to their wide substrate specificity and lack of clinically available inhibitors.[2] MBLs are classified into three subclasses, B1, B2, and B3, according to the set of metal ion ligands in the active center.[3] Most recently, a new metallo-β-lactamase has been found in India, Pakistan, and also in the United Kingdom[4] and many other countries. Further proliferation of the types of metallo-β-lactamases is becoming a grave worldwide public health concern.[5] Among the currently known MBLs, IMP-1, belonging to subclass B1, is one of the most serious threats because the gene encoding it is located in an integron structure on a plasmid,[6] which is horizontally transferable between bacterial strains.

The X-ray crystal structure of IMP-1 has been determined and two Zn^{II} ions (termed Zn1 and Zn2) were found in the active center.[7] Zn1 is tetrahedrally coordinated by His116, His118, and His196, whereas Zn2 is trigonal–pyramidally coordinated by Asp120, Cys221, His263, and a water molecule (Figure 1). In addition, OH_2 or OH^- is thought to be bridged between Zn1 and Zn2, although no electron density for this molecule is observed due to the low resolution of the crystal structure.

Correlation between Zn^{II} content and catalytic efficiency continues to be of great controversy,[8] despite the fact that Zn^{II} ligands are well-conserved in other subclass B1 MBLs, for example, BcII from *Bacillus cereus* and CcrA from *Bacteroides fragilis*.[9] BcII has one Zn^{II} ion under physiological conditions but achieves maximum activity when two Zn^{II} ions are bound to the active center. Unlike BcII, CcrA and IMP-1 contain two Zn^{II} ions under physiological conditions and require two Zn^{II} ions for catalysis.

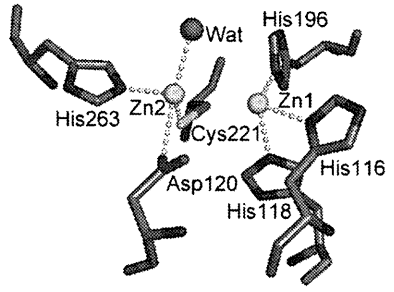

Figure 1. Schematic representation of the active site of IMP-1 from *Pseudomonas aeruginosa* (PDB ID: 1DDK). The Zn^{II} (Zn1, Zn2) atoms and a water (Wat) molecule are shown as spheres.

To investigate the metal coordination environment in the active center, spectroscopic studies on Co^{II}-substituted MBLs have been extensively carried out, due to the spectroscopically silent Zn^{II}.[10] Success in these studies requires preparation of apo-enzymes. In BcII and CcrA, demetallations of Zn^{II} and metal substitution with Co^{II} have been performed successfully.[10] Similar efforts have been attempted for IMP-1, but complete demetallation with various chelating agents, such as EDTA, yields samples that cannot be reactivated in the presence of excess Zn^{II} ions.[11]

Herein, we describe the kinetic behavior of the inactivation of IMP-1 by EDTA, and successful preparation of the apo-enzyme (apo-IMP-1). In addition, spectroscopic characterization of Co^{II}-substituted IMP-1 derived from apo-IMP-1 with Co^{II}, in situ, is also described.

Temperature dependency for inactivation of IMP-1 (10 μM) by EDTA (50 mM) in MOPS (50 mM; pH 7.0) and NaCl (1.0 M) was examined by changing the temperature of the incubation samples (0, 10, 20, and 30 °C, Figure S1 in the Supporting Information). Figure 2 shows the plot of the residual activity against incubation time at 10 °C, where the residual activity, k_t/k_0, (i.e., the relative activity) is the ratio of the molecular activity, k_t, of the activated IMP-1 at each incubation time of IMP-1 with EDTA to the molecular activity, k_0, of IMP-1 before the addition of EDTA at $t=0$ (control).

From Figure 2, it is apparent that the inactivation reaction proceeds in two steps, namely fast and slow. A plausible explanation for this biphasic behavior is successive demetallation of the two Zn^{II} ions from the active center (Scheme 1).

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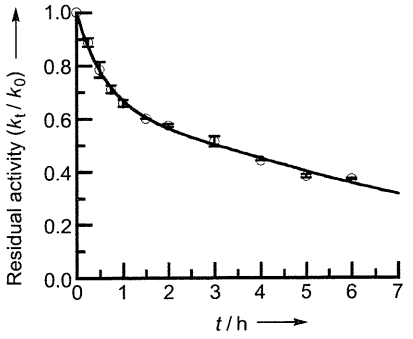
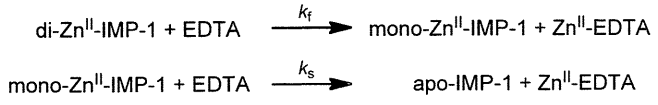


Figure 2. Residual activity of IMP-1 (10 μM) against time in MOPS-NaOH (50 mM, pH 7.0) and NaCl (1.0 M) at 10 °C in the presence of EDTA (50 mM). Values are the mean ± S.D. from three independent experiments performed in duplicate. The solid line was generated by a nonlinear least-squares fit of the data to Equation (1), as described in the text.



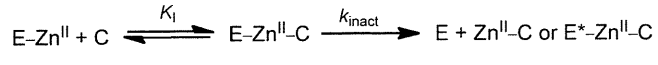
Scheme 1. Successive demetallation of the two Zn^{II} ions from the active center of IMP-1.

Assuming that the kinetics of demetallation from IMP-1 obey two pseudo-first-order reactions (Equations (S1)–(S6) in the Supporting Information), the residual activity can be expressed by Equation (1), where k_f and k_s are the rate constants for the fast and slow inactivation reactions, respectively:

$$\frac{k_t}{k_0} = \exp(-k_f t) + \frac{k_f}{k_s - k_f}[\exp(-k_f t) - \exp(-k_s t)] \times \alpha \qquad (1)$$

We estimated the following rate constants and α for inactivation of IMP-1 with EDTA at 10 °C using a nonlinear least-squares fitting of the experimental data to Equation (1): $k_f = 1.26(\pm 0.06)\ h^{-1}$, $k_s = 0.12(\pm 0.01)\ h^{-1}$, and $\alpha = 0.67 \pm 0.04$. At temperatures other than 0 °C, however, the transition from the faster phase to the slower phase could not clearly be discriminated (Figure S1 in the Supporting Information).

Inactivation of IMP-1 with EDTA is a complicated process. Laraki et al. have suggested that the inactivation of IMP-1 by a metal ion chelator follows a pseudo-first-order reaction and have proposed the mechanism shown in Scheme 2, where E*–Zn^{II}–C represents the inactive ternary complex of the enzyme–Zn^{II}–chelator.[12] They found that EDTA is a poor inactivator because only 10% of the activity was lost after 1 h with 10 mM chelator in HEPES (50 mM, pH 7.5).[12] Siemann et al. also described the inactivation of IMP-1 with chelators based on Scheme 2, but the inactivation was not reversible except in the case of one chelator (zincon).[11b] The treatment of IMP-1 with dipicolinic acid resulted in production of the mono-Zn^{II}–IMP-1, as determined by electrospray ionization mass spectrometry under nondenaturing conditions. The k_{cat} value of mono-Zn^{II}–IMP-1 (137 s^{-1}) for nitrocefin as the substrate was approximately half that of di-Zn^{II}–IMP-1 (260 s^{-1}); this indicates that the second equivalent of Zn^{II} enhances enzyme catalytic activity.[11b]



Scheme 2. The mechanism for the inactivation of IMP-1 by a metal ion chelator according to the proposed pseudo-first-order reaction.[12] E*–Zn^{II}–C is the inactive ternary complex of the enzyme–Zn^{II}–chelator.

In this study, the relative ratio of the activity of mono-Zn^{II}–IMP-1 to that of di-Zn^{II}–IMP-1, α, was found to be 0.67. This indicates that the activity of mono-Zn^{II}–IMP-1 is 67% that of the parent di-Zn^{II}–IMP-1 and that the two Zn^{II} ions have different binding affinities for IMP-1. Based on the results described above, we attempted to prepare apo-IMP-1 by the application of relatively high temperature and high enzyme concentration (see the Supporting Information). A typical procedure is as follows. A solution of EDTA (200 mM) in MOPS-NaOH (50 mM, pH 6.5) and NaCl (1.0 M) was added drop-wise to the slowly stirred IMP-1 solution (1.5 mM) in MOPS-NaOH (50 mM, pH 6.5), NaCl (1.0 M), and glycerol (30%). The final concentration of EDTA was adjusted to 50 mM. After incubation at 30 °C and centrifugation, the supernatant was passed through a PD-10 column at 4 °C. This method yielded preparations with 0.025 Zn^{II} per molecule of IMP-1, as checked by atomic absorption spectrometry, and the activity of apo-IMP-1 was less than 5% that of native IMP-1. The addition of excess $Zn(NO_3)_2$ to apo-IMP-1 showed activity comparable to that of the native enzyme (Table S1 in the Supporting Information). When 2 equiv Zn^{II} was added to apo-IMP-1, the CD spectrum of reconstituted IMP-1 was consistent with that of native IMP-1 (Figure 3A); this indicates that the helix content does not change and the secondary structure in the protein is maintained.

Preliminary differential scanning calorimetry measurements also showed an increase of the denaturation temperature from 56.4 °C for apo-IMP-1 or 56.5 °C for mono-Zn^{II}–IMP-1 (apo-IMP-1 + 1 equiv Zn^{II}) to 76.9 °C for native IMP-1 (Figure 3B). These findings suggest that the second equivalent of Zn^{II} contributes both to enhance catalytic activity and structural stability in the Zn^{II} coordination sphere during the catalytic cycle, which can help to achieve maximum activity.

In contrast, the preparation of apo-IMP-1 by a dialysis method with EDTA at 4 °C took about 143 h, and was unsuccessful due to irreversible denaturation. Haruta et al. also reported the preparation of apo-IMP-1 by three days of dialysis of the EDTA-treated enzyme against Zn^{II}-free MOPS, but only 30% of its original activity was restored, even after addition of 1 mM $Zn(NO_3)_2$ to apo-IMP-1.[11a]

To extrapolate the coordination environment of mono-Zn^{II}– and di-Zn^{II}–IMP-1, spectrophotometric titration of prepared apo-IMP-1 with $CoCl_2$ was carried out in MOPS-NaOH (50 mM, pH 7.0), NaCl (1.0 M), and glycerol (30%) under argon. Figure 4A shows the UV-visible difference spectral changes when $CoCl_2$ was added, up to 2 equiv in 0.2 equiv increments (the spectrum of apo-IMP-1 was subtracted from each spectrum of

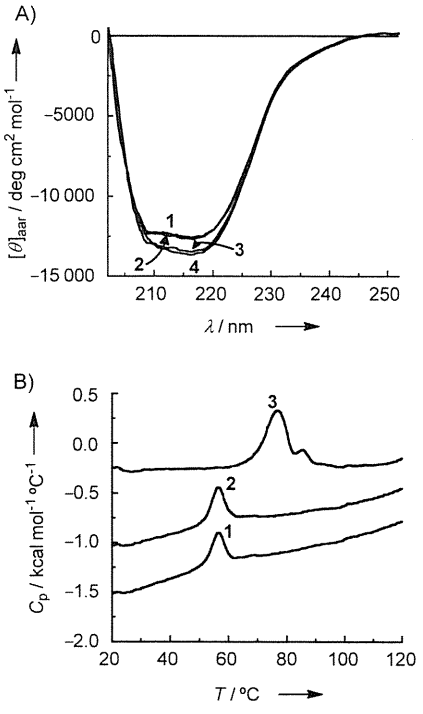


Figure 3. A) CD spectra of apo-IMP-1 (**1**), mono-Zn^{II}–IMP-1 (**2**; apo-IMP-1 + 1 equiv Zn^{II}), native IMP-1 (**3**), and di-Zn^{II}–IMP-1 (**4**; apo-IMP-1 + 2 equiv Zn^{II}) in MOPS-NaOH (50 mM, pH 7.0), NaCl (1.0 M), and glycerol (30%) at 25 °C, and B) DSC transitions of apo-IMP-1 (**1**; 34 μM), mono-Zn^{II}–IMP-1 (**2**; apo-IMP-1 + 1 equiv Zn^{II}; 38 μM), and native IMP-1 (**3**; 36 μM) in potassium phosphate (50 mM, pH 7.0), NaCl (1.0 M), and glycerol (30%).

the Co^{II} added enzyme). For concentrations up to 1 equiv of Co^{II}, an intense absorption band was observed at 350 nm, which was attributed to the thiolate group of Cys221→Co^{II} ligand-to-metal charge transfer (LMCT) band. Concurrently, d–d transitions in the range of 500–650 nm were also observed. Further addition of 1 equiv Co^{II} to mono-Co^{II}–IMP-1 gave the LMCT band at 350 nm (difference molar extinction coefficient, $\varepsilon = 1200\ M^{-1}cm^{-1}$) and four readily distinguishable d–d transitions at 520 (280), 556 (360), 612 (250), and 635 nm (210).

The molar extinction coefficient increased with increasing concentration of Co^{II} and reached a plateau when the Co^{II} content exceeded 2 equiv (Figure 4A, inset), but preferential absorption at either 350 or 500–650 nm was not observed. The molar extinction coefficient at 350 nm in di-Co^{II}–IMP-1 is about threefold higher than that in mono-Co^{II}–IMP-1. Although more accurate estimation of the dissociation constants by competition experiments with chelators, such as Mag-fura-2, could be performed, the macroscopic dissociation constants were estimated by fitting of the titration data by using the program DynaFit,[13] and found to be $K_{D1} < 60$ nM and $K_{D2} = 0.3$ μM (Figure 4A, inset, and Table S2 in the Supporting Information), which are close to those of BcII (Table S2 in the Supporting Information). No precipitation was observed even up to the addition of 3 equiv of $CoCl_2$ to apo-IMP-1. In contrast, de Seny et al. and Periyannan et al. reported that the addition of Co^{II} to apo-BcII and apo-CcrA resulted in the formation of a precipitate.[10b,d]

Assignment of the coordination geometries by absorption and CD spectra have proven difficult for Co^{II} complexes and Co^{II}-substituted metalloenzymes. The room temperature magnetic circular dichroism (MCD) spectra in conjunction with absorption spectra provide valuable information in defining the overall coordination geometries of Co^{II} coordination sites more accurately.[14] The room temperature MCD spectra of apo-IMP-1 containing 1.0 and 2.0 equiv Co^{II} in MOPS-NaOH (50 mM, pH 7.0), NaCl (1.0 M), and glycerol (30%) are shown in Figure 4C. The addition of 1.0 equiv of Co^{II} to apo-IMP-1 resulted in two extrema at 330(−) nm and 360(+) nm, derived from a Cys221→Co^{II} LMCT band, and a broad negative extrema near 550 nm. The addition of 2 equiv of Co^{II} to apo-IMP-1 gave rise to six distinct extrema at 335(−), 360(+), 520(+), 556(−), 612(−), and 635(−) nm, with a substantial increase in the intensity, which is quite similar to that of the pentacoordinate Co^{II}-substituted β-lactamase II.[10h]

For detailed examination of the Co^{II} coordination sphere in Co^{II}-substituted IMP-1, the C221A mutant in which cysteine at position 221 is replaced by alanine was over-expressed and purified. This apo-enzyme (apo-C221A) was then prepared according to our procedure described above (see the Supporting Information).

The UV/Vis difference spectrum (Co^{II}-bound apo-C221A) of apo-C221A containing 1 equiv Co^{II} in MOPS-NaOH (50 mM, pH 7.0), NaCl (1 M), and glycerol (30%) exhibited an absorption maximum at 550 nm ($\varepsilon = 130\ M^{-1}cm^{-1}$, Figure 4B). The macroscopic dissociation constant was estimated by fitting of the titration data, and found to be $K_{D1} = 2.4$ μM (Figure 4B, inset, and Table S2 in the Supporting Information). The corresponding MCD spectrum showed an intense negative extremum between 500 and 600 nm (Figure 4D). This MCD spectrum is analogous to those of tetrahedral Co^{II} enzymes, such as the acidic form of carbonic anhydrase;[14] this indicates that Co^{II} in mono-Co^{II}–C221A is likely to occupy the Zn1 site with tetracoordinate Co^{II} geometry. No LMCT band from Cys221→Co^{II} was observed. Further addition of 1 equiv of Co^{II} to mono-Co^{II}–C221A resulted in a spectral change with a similar shape. From a comparison of the UV/Vis difference and MCD spectra between IMP-1 and the C221A mutant, the d–d transitions in the range of 500–600 nm are mainly due to metal binding at the Zn1 site, but those at 612 and 635 nm are partially attributed to metal binding at the Zn2 site. Moreover, the results of our spectroscopic data suggest that Co^{II} in mono-Co^{II}–IMP-1 might be distributed between both the Zn1 and Zn2 sites with a ratio of about 2:1. These results are supported by the prediction for BcII by de Seny et al.,[10b] in which the metal ion is distributed between both sites when the apo-enzyme is reconstituted with 1 equiv of Co^{II} or Cd^{II}.

In conclusion, we have established a new preparation method for generating the apo-enzyme of IMP-1, in which the activity can be restored by the addition of Zn^{II} ions. Key to the successful preparation of apo-IMP-1 are: 1) inclusion of 1.0 M NaCl and more than 10% glycerol in buffer, 2) demetallation for a short time (~12 h) at mild temperature (30 °C) without di-

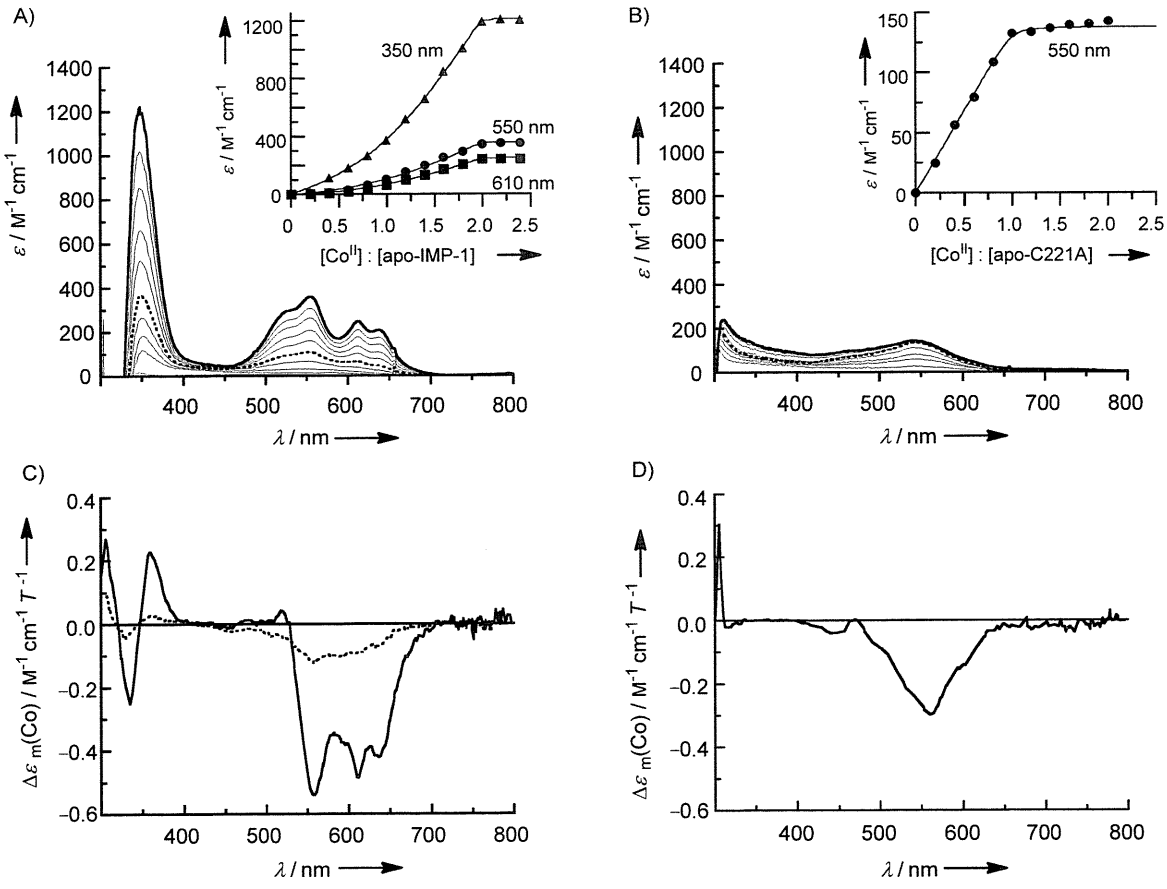


Figure 4. UV–visible difference and MCD spectra of apo-IMP-1 and apo-C221A with Co^{II}. A) apo-IMP-1 (633 μM) in MOPS-NaOH (50 mM, pH 7.0), NaCl (1.0 M), and glycerol (30%) was titrated with 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0 equiv of Co^{II}. The thick broken and solid lines show the addition of Co^{II} to apo-IMP-1 up to 1 and 2 equiv, respectively. The spectrum of apo-IMP-1 was subtracted from each spectrum of the Co^{II}-added enzyme. Inset: plot of the difference molar extinction coefficient (ε) at 350, 550, and 610 nm as a function of Co^{II} equivalents added to apo-IMP-1. The solid lines correspond to a sequential two-step binding model (see the Supporting Information) by using the program DynaFit (BioKin, Ltd.).[13] The dissociation constants obtained were K_{D1} < 60 nM and K_{D2} = 0.3 μM. B) apo-C221A (516 μM) in MOPS-NaOH (50 mM, pH 7.0), NaCl (1.0 M), and glycerol (30%) was titrated with 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0 equiv of Co^{II}. The thick broken and solid lines show the addition of Co^{II} to apo-C221A up to 1 and 2 equiv, respectively. The spectrum of apo-C221A was subtracted from each spectrum of the Co^{II}-added enzyme. Inset: plot of the difference molar extinction coefficient (ε) at 550 nm as a function of Co^{II} equivalents added to apo-C221A. The solid line corresponds to a one-step binding model (see the Supporting Information) by using the program DynaFit.[13] The dissociation constant obtained was K_{D1} = 2.4 μM. C) The room temperature MCD spectra of apo-IMP-1 (510 μM) upon the addition of 1 equiv (-----) and 2 equiv (——) Co^{II} in MOPS-NaOH (50 mM, pH 7.0), NaCl (1.0 M), and glycerol (30%). D) The room temperature MCD spectra of apo-C221A (650 μM) upon addition of 2 equiv Co^{II} in MOPS-NaOH (50 mM, pH 7.0), NaCl (1.0 M), and glycerol (30%).

alysis, and 3) choice of desalting column. Based on the success of this apo-enzyme preparation, the spectroscopic properties of apo-IMP-1 with Co^{II} ions could then be investigated. Finally, this method can be used to prepare not only apo-IMP-1, but also other metallo-β-lactamases. Indeed, the apo-enzyme of IND-7 metallo-β-lactamase could be prepared easily.

Acknowledgements

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Keywords: antibiotics · bioinorganic chemistry · lactams · metalloenzymes · UV/Vis spectroscopy

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Tuberculosis infection among homeless persons and caregivers in a high-tuberculosis-prevalence area in Japan: a cross-sectional study

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Abstract

Background: Tuberculosis (TB) is a major public health problem. The Airin district of Osaka City has a large population of homeless persons and caregivers and is estimated to be the largest TB-endemic area in the intermediate-prevalence country, Japan. However, there have been few studies of homeless persons and caregivers. The objective of this study is to detect active TB and to assess the prevalence and risk factors for latent TB infection among homeless persons and caregivers.

Methods: We conducted a cross-sectional study for screening TB infection (active and latent TB infections) using questionnaire, chest X-ray (CXR), newly available assay for latent TB infection (QuantiFERON-TB Gold In-Tube; QFT) and clinical evaluation by physicians at the Osaka Socio-Medical Center Hospital between July 2007 and March 2008. Homeless persons and caregivers, aged 30-74 years old, who had not received CXR examination within one year, were recruited. As for risk factors of latent TB infection, the odds ratios (OR) and 95% confidence intervals (95% CI) for QFT-positivity were calculated using logistic regression model.

Results: Complete responses were available from 436 individuals (263 homeless persons and 173 caregivers). Four active TB cases (1.5%) among homeless persons were found, while there were no cases among caregivers. Out of these four, three had positive QFT results. One hundred and thirty-three (50.6%) homeless persons and 42 (24.3%) caregivers had positive QFT results. In multivariate analysis, QFT-positivity was independently associated with a long time spent in the Airin district: ≥10 years versus <10 years for homeless (OR = 2.53; 95% CI, 1.39-4.61) and for caregivers (OR = 2.32; 95% CI, 1.05-5.13), and the past exposure to TB patients for caregivers (OR = 3.21; 95% CI, 1.30-7.91) but not for homeless persons (OR = 1.51; 95% CI, 0.71-3.21).

Conclusions: Although no active TB was found for caregivers, one-quarter of them had latent TB infection. In addition to homeless persons, caregivers need examinations for latent TB infection as well as active TB and careful follow-up, especially when they have spent a long time in a TB-endemic area and/or have been exposed to TB patients.

Background

Globally, there were an estimated 9.27 million cases of tuberculosis (TB) in 2007, with the larger number of cases of latent TB infection [1]. The Airin district of Osaka City is known as an urban area with a dense population of day-laborers and homeless people in Japan, an intermediate-prevalence country [2]. The prevalence of active TB in the Airin district was approximately 1,000 per 100,000 in the early 2000s, which was the highest in Japan and similar to that in developing countries [1,3,4]. In the Airin district, there are estimated to be 15,000 to 20,000 homeless persons with no medical insurance, accounting for 80% of visitors to a free or low-cost hospital, i.e. Osaka socio-medical center

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hospital [3]. TB screening programs have been carried out at Airin Health Office since 1990 and at the Osaka socio-medical center hospital since 2005. Mobile screening for TB was carried out monthly between 1973 and 2005, and weekly since 2006, by the Osaka City government on the streets of the Airin district [5]. The directly observed therapy short course (DOTS) program for homeless persons has been carried out since 1999. To date, several communities (i.e., NGOs, hospitals and facilities) for accommodation, health and welfare have been organized for homeless persons, following the Stop TB Strategy [5,6]. The prevalence of active TB in Osaka City decreased by 50% from 2000 to 2008. However, there are still estimated to be many unknown active TB cases in the Airin district [5].

Previous studies have identified risk factors for TB, such as immigrants, HIV, poverty, incarceration, smoking and alcohol use [7-10]. Homelessness-related TB remains a widespread problem [7]. An anti-TB strategy targeting homeless people, empowering caregivers and communities, and promoting research has been recommended [6,7]. However, studies on TB infection of homeless persons and their caregivers are scarce [11-13].

The commercially available blood test, QuantiFERON-TB Gold In-Tube (QFT; Cellestis Limited, Carnegie, Australia), is an interferon-gamma release assay (IGRA) in response to *M. tuberculosis*-specific antigens [14], and has been validated [15]. The QFT has excellent specificity and gives us valuable information of latent TB infection, even for a Japanese Bacillus Calmette-Guérin (BCG)-vaccinated population, whereas the accuracy of the tuberculin skin test (TST) is hampered by poor specificity due to the widespread use of BCG vaccination and re-vaccination in Japan [16].

The principal mechanism for the Stop TB Strategy is the detection and treatment of patients with TB [1]. The objectives of this study were to detect active TB cases and to assess the prevalence and risk factors of latent TB infection among homeless persons and their caregivers in a TB-endemic area in an intermediate-prevalence country.

Methods

Participants and Measurements

We conducted a cross-sectional study of homeless persons and their caregivers in the Airin district. Homeless persons were defined as persons who had had no permanent residence for more than one month. Caregivers were defined as persons who worked and supported homeless persons with regard to job arrangement, medical care, food supply, accommodation and clothing in the Airin district. Caregivers who belonged to five NGOs (mainly associated with a homeless shelter, job assistance, anti-alcoholism action, a soup-run and DOTS), as well as staff at the Osaka socio-medical center hospital and two clinics (nurses, social workers, dietitians, counselors and physicians) were enrolled in this study. Through our network of five NGOs, Osaka socio-medical center hospital and two local clinics by the use of posters, handouts and personal communication through a study recruiter, homeless persons and caregivers aged 30-74 years who had not received chest X-ray (CXR) examination within one year were recruited for tuberculosis screening at Osaka socio-medical center hospital between July 2007 and March 2008.

CXR, QFT, questionnaire and clinical evaluation by physicians were performed. If a participant had the symptom of sputum, smear testing and culture of sputum were performed for active TB diagnosis. A physician's interview using a standardized questionnaire covered the following data: age, sex, past history of TB, use of immunosuppressive drugs, past exposure to patients diagnosed with tuberculosis, current smoking and drinking status (more than 10 g of ethanol almost every day, yes or no), present symptoms of cough and/or sputum, general fatigue, elevated body temperature (≥37.0°C) and years spent living and/or working in the Airin district. Past exposure to tuberculosis patients was defined as self-reported exposure through living and/or working with TB patients in a shared space before the patients had been diagnosed with active TB.

CXR findings were categorized into two groups, normal and abnormal, based on our standard method [4]. Further classification was not performed since TB shadows vary and could take any kind of shape [17]. Quality control was performed by double-checks on each radiograph by another TB specialist. Active TB case was defined as an individual with symptoms compatible with TB plus detection of nucleic acid from mycobacterium tuberculosis complex from a clinical specimen, or as a patient with tuberculosis clinically suspected by an expert physician plus a response to anti-tuberculosis treatment [18].

QFT was performed and interpreted according to the manufacturer's instructions, with an interferon-γ response for the tuberculosis antigen tube minus Nil of ≥0.35 IU/ml defined as a positive result. QFT results were considered indeterminate if the subject did not respond to the mitogen-positive control tube with at least 0.5 IU/ml of interferon-γ [19,20].

Written informed consent was provided by each participant and each received 500 yen (about five US dollars) as an incentive to participate. The ethical committees of Osaka socio-medical center hospital and Osaka University approved this study.

Statistical Analysis

The proportions of basic characteristics and TB-related findings were descriptively shown among homeless persons and caregivers. The prevalence and 95% confidence

intervals (95% CI) for active TB were calculated using method based on the F-distribution.

The odds ratios (OR) and 95% CI for QFT-positivity were calculated using logistic regression model. We assessed how the positive QFT prevalence varied according to the potential factors such as length of time spent living and/or working in the Airin district and past exposure to TB patients, using the dichotomized categories to avoid statistical instability derived from small sample size. We investigated the multivariate model among homeless persons and caregivers, who did not have active TB disease or indeterminate QFT result.

Because of the failure in convergence, we removed the variables that had <5% cases with exposure or non-exposure category from the age-adjusted and multivariate logistic model [21]. Thereby, female and elevated body temperature for homeless, cough and/or sputum, elevated body temperature, past history of TB and abnormal chest X-ray finding for caregivers were excluded from the analyses.

Probability values for statistical tests were two-tailed and $p < 0.05$ was regarded as statistically significant. The SAS statistical software package (version 9.1; SAS Institute Inc., Cary, NC, USA) was used for all analyses.

Results

A total of 448 persons were enrolled in the study. However, of these 448 participants, four homeless persons refused to give a blood sample, and eight homeless persons left the waiting room before having a CXR and/or completing a questionnaire. Thus, complete results of CXR, QFT and questionnaire were available for 436 participants (263 homeless persons and 173 caregivers).

Table 1 shows the basic characteristics and TB-related findings for homeless persons and caregivers. The homeless persons included only two women and exhibited higher values for almost all variables of interest than caregivers. Four active TB cases were found among the homeless persons: the prevalence of 1.52% (95% CI, 0.42-3.85), while there were no cases among caregivers. One-half of the homeless persons (50.6%) and one-quarter of the caregivers (24.3%) had a positive QFT result.

Table 2 shows the characteristics of active TB cases. Out of the four active TB cases, all four subjects were homeless, male, more than 60 years of age, had abnormal CXR findings, had lived or worked for more than 9 years (mean 13 years) in the Airin district and had symptoms of cough and/or sputum. Three subjects had positive QFT results. None of them had acknowledged past exposure to TB patients.

Table 3 shows age-adjusted and multivariate OR (95% CI) of QFT-positivity according to potential risk factors among homeless persons and caregivers, who did not have active TB disease or indeterminate QFT result. Both homeless persons and caregivers who had spent more than ten years in the Airin district had significantly higher positive QFT result than those who had spent less than ten years in both age-adjusted and multivariate models. When we used a cutoff point of half of the period, namely, five years, homeless persons who had spent more than five years had significantly higher positive QFT result (multivariate odds ratio, 2.93; 95% CI, 1.43-6.01) but caregivers did not (multivariate odds ratio, 1.93; 95% CI, 0.84-4.45). Current drinker was a significant predictive factor for QFT-positivity among homeless persons, but not among caregivers in both age-adjusted and multivariate models. The caregivers who had acknowledged past exposure to TB patients had significantly higher QFT-positivity than those who did not in both age-adjusted and multivariate models, while the homeless persons did not in the multivariate model.

Past history of TB and abnormal chest X-ray finding were not associated significantly with positive QFT result among homeless persons in the multivariate model, and the analysis was not carried out for caregivers owing to the small number with a past history or abnormal chest X-ray finding.

Discussion

Active TB prevalence

We showed that homeless people in the Airin district remain at high risk for active TB, and that the cross-sectional estimate of the prevalence of active TB was a rate of 1.52% (95% CI, 0.42-3.85) among homeless people in the Airin district, Osaka, Japan. A similar high prevalence of active TB was reported among homeless day-laborers (2.2%) in the same district from a previous study in 2003-2005 [4]. According to annual TB patients' registry database, the prevalence of active TB among residents in the Airin district was 653 per 100,000 (0.65%) in 2007, and the homeless people accounted for 79.3% of TB patients in that district [22]. These estimates of active TB prevalence were markedly higher than those of the total Japanese population and the Osaka City population in 2007, namely, 19 per 100,000 (0.019%) and 53 per 100,000 (0.053%), respectively [1,22].

High prevalence of active TB among the homeless population was reported from other countries. In New York City, McAdam et al. reported the high active TB prevalence of 1,502 per 100,000 among homeless persons in 1992 [23]. In London, Story et al. showed the high prevalence of 788 per 100,000 among homeless people in 2003 [24]. TB is concentrated in the homeless population in developed countries and TB transmission may occur from homeless people to other populations [25].