Supplementary Data

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Detection of a Metallo-β-Lactamase (IMP-1) by Fluorescent Probe having Dansyl and Thiol groups

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Experimental procedures

Materials. All chemicals were obtained commercially and were of the highest quality available, and were used without further purification. Dansylethylenediamine was prepared by reacting of dansyl chloride with 10 equimolar amounts of ethylenediamine in benzene at 70 ºC for 2h.

Methods. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were recorded on a JEOL α-500 instrument; δ values are given in ppm relative to tetramethylsilane. COSY, HMQC, and HMBC experiments have been carried out to assign the ^{1}H and ^{13}C spectra completely. IR spectra were recorded on a JEOL JIR 6500W spectrophotometer. UV spectra were recorded on a Shimadzu UV-2200 spectrophotometer, using 1-cm quartz cells. Mass spectra (MS) were measured with a JEOL JMS-DX303HF spectrometer.

Production and purification of wild-type IMP-1 metallo-β-lactamase. Wild-type IMP-1 metallo-β-lactamase was produced and purified according to the published method.[SR1] The concentration of the purified enzyme was determined by measuring the absorption at 280 nm of enzyme preparation using an extinction coefficient of 4.9×10^4 $M^{-1}cm^{-1}$.

Preparation of apo-enzyme and cobalt(II)-substituted enzyme. To a 1.55 mM solution of IMP-1 in 50 mM MOPS-NaOH (pH 6.5) containing 30 % glycerin and 1.0 M NaCl was added 50 mM EDTA in 50 mM MOPS-NaOH (pH 6.5) containing 1.0 M NaCl under argon.

The enzyme solution was sealed under argon and kept to stand for 15 h at 30 ºC. After activity of hydrolysis was less than 5 % compared with that of control EDTA, the reaction mixture was centrifuged at 20000 × g for 40 min at 4 ºC to remove precipitates. The supernatant was passed through a PD-10 column at room temperature (eluent, 50 mM MOPS-NaOH (pH 7.0), 1.0 M NaCl, 30 % glycerin buffer prepared with demetallated water (Wako chemical Co., LTD)). The activity of apo-emzyme was < 5 % that of the holoenzyme. The Co(II)-substituted enzyme was prepared by addition of 2 equimolar amounts of $CoSO_4 \bullet 7H_2O$ in small portion to a buffered solution of apo-enzyme at room temperature.

Fluorometric Analysis. Fluorescence excitation was either at 280 nm (tryptophan excitation) or 340 nm (DansylC2SH excitation). Emission spectra were recorded either from 300 nm to 900 nm (for 280-nm excitation) or from 350 nm to 900 nm (for 340-nm excitation). Fluorescence quantum yield (Φ) were based on quinine sulfate having a quantum yield of 0.54 in 0.1 M sulfuric acid.

Fluorometric titrations. For a typical titration, 30 μL of a 100 μM methanolic stock solution of DansylC2SH was added to 2.7 mL of 50 mM Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl and 10 % methanol. Subsequently, 6 μL of a 50 μM stock solution of the IMP-1 metallo-β-lactamase in 50 mM Tirs-HCl buffer (pH 7.4) containing 0.5 M NaCl was added to the former solution (0–10 μM total IMP-1). The fluorescence intensity of each solution was measured using a Hitachi F-4500 fluorescence spectrophotometer with 5 nm bandwidth and 1-cm quartz, exciting at 340 nm and recording emission from 350 nm to 900 nm.

Determination of apparent dissociation constant with IMP-1. The dissociation equilibrium is defined by

$$\text{DansylC2SH-IMP-1} \overset{K_D}{\rightleftharpoons} \text{DansylC2SH + IMP-1} \tag{1}$$

and with dissociation constant

$$K = \frac{[\text{DansylC2SH}]_{\text{free}}[\text{IMP-1}]_{\text{free}}}{[\text{DansylC2SH-IMP-1}]} \tag{2}$$

where $[\text{DansylC2SH}]_{\text{total}} = [\text{DansylC2SH}]_{\text{free}} + [\text{DansylC2SH-IMP-1}]$ (3)

and $[\text{IMP-1}]_{\text{total}} = [\text{IMP-1}]_{\text{free}} + [\text{DansylC2SH-IMP-1}]$ (4)

The overall molar fluorescence intensity F is defined by

$$F = \mathrm{a}\frac{[\text{DansylC2SH-IMP-1}]}{[\text{DansylC2SH}]_{\text{total}}} + \mathrm{b}\frac{[\text{DansylC2SH}]_{\text{free}}}{[\text{DansylC2SH}]_{\text{total}}} \tag{5}$$

where a is the maximum fluorescence intensity and b is the fluorescence intensity with no addition of IMP-1, respectively.

Equations 2-4 are substituted in equation (5) and then equation (6) is obtained.

$$F = \mathrm{a}\frac{K_\mathrm{D} + [\mathrm{IMP}\text{-}1]_\mathrm{total} + 1 - \sqrt{(K_\mathrm{D} + [\mathrm{IMP}\text{-}1]_\mathrm{total} + 1)^2 - 4[\mathrm{IMP}\text{-}1]_\mathrm{total}}}{2} + \mathrm{b}\frac{1 - K_\mathrm{D} - [\mathrm{IMP}\text{-}1]_\mathrm{total} + \sqrt{(K_\mathrm{D} + [\mathrm{IMP}\text{-}1]_\mathrm{total} + 1)^2 - 4[\mathrm{IMP} - 1]_\mathrm{total}}}{2} \quad (6)$$

where $[\mathrm{IMP}\text{-}1]_\mathrm{total}$, a and b were set at 1.0 μM, 9.01, and 1.9, respectively.

The apparent dissociation constant, K_D, was fitted to equation 6 using non-linear least-square method (KaleidaGraph software).[SR2]

Kinetics Measurements and Inhibition Study. Hydrolysis of a substrate, nitrocefin, was monitored at 491 nm using a Hitachi U 1500 spectrophotometer in a total volume of 3.1 mL. The 50 % inhibition concentration (IC_{50}) for IMP-1 was determined as following: the IMP-1 metallo-β-lactamase was preincubated with DansylC2SH for 10 min at 30 ºC in 50 mM Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl. Initial velocities were measured after the addition substrate, nitrocefin, at approximate K_M concentration (20 μM for the IMP-1 metallo-β-lactamase determined in this laboratory).

Nine stock solutions of DansylC2SH in methanol (15.5 μM, 31.0 μM, 77.5 μM, 155 μM, 310 μM, 775 μM, 1.55 mM, 2.33 mM, and 31.0 mM) were prepared. 1.0 mL of each stock solution and 0.1 mL of 31 nM of IMP-1 dissolved in 50 mM Tris-HCl buffer (pH7.4) containing 0.5 M NaCl were placed in the cuvette filled with a 2.8 mL of 50 mM Tris-HCl buffered (pH7.4) solution containing 0.5 M NaCl. The mixture was preincubated for 10 min at 30 ºC. 0.1 mL of a 3.1 mM nitrocefin solution in DMSO was added and the changes in absorbance at 491 nm was recorded for 3 min at 30 ºC.

Final concentration of DansylC2SH was 0.5, 2.5, 5, 25, 50, 75, and 100 μM in each measurement.

In control experiment was used 0.1 mL of methanol instead of DansylC2SH solution under the same conditions.

From the obtained initial velocities, the apparent molecular activity, k_2 /min^{-1}, = v/[IMP-1] was determined and was plotted as a function of the concentration of DansylC2SH.

The inhibition constant, K_i, for the IMP-1 metallo-β-lactamase was determined by fitting the data to competitive inhibition pattern (equation 7) using non-linear least-square method (KaleidaGraph software).[SR2]

$$v = V_{max}[S]/\{K_M(1+[I]/K_i)+[S]\} \quad (7)$$

where V_{max} is the maximum velocity and K_M is Michaelis constant, and [S] and [I] are the concentrations of nitrocefin and inhibitor, respectively. V_{max}, [S], and K_M are set at 27000, 100 μM, and 20 μM.

Supporting References:

(SR1) M. Goto, T. Takahashi, F. Yamashita, A. Koreeda, H. Mori, M. Ohta and Y. Arakawa, *Biol. Pharm. Bull.*, 1997, **20**, 1136.

(SR2) KaleidaGraph (1997) Synergy Software Inc., 2457 Perkiomen Ave., Reading, PA 19606.

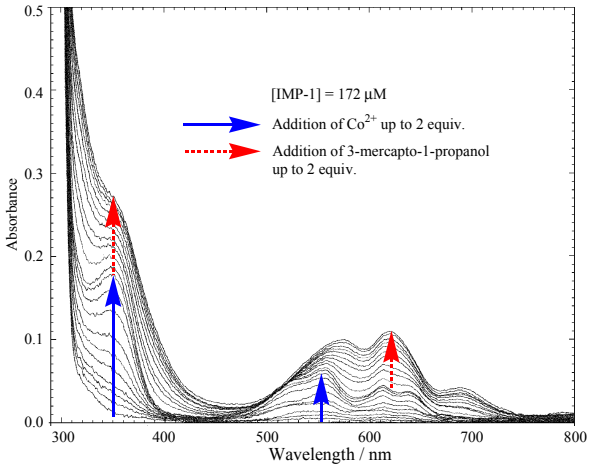

Figure S1. Visible spectral change of apo-β-lactamase with varying concentration of Co^{2+} and Co^{2+}-substituted β-lactamase by titration of 3-mercapto-1-propanol.