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Biochemical Characterization of Sfh-I, a Subclass B2 Metallo-β-Lactamase from *Serratia fonticola* UTAD54[▽]

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The subclass B2 metallo-β-lactamase (MBL) Sfh-I from *Serratia fonticola* UTAD54 was cloned and overexpressed in *Escherichia coli*. The recombinant protein binds one equivalent of zinc, as shown by mass spectrometry, and preferentially hydrolyzes carbapenem substrates. However, compared to other B2 MBLs, Sfh-I also shows limited hydrolytic activity against some additional substrates and is not inhibited by a second equivalent of zinc. These data confirm Sfh-I to be a subclass B2 metallo-β-lactamase with some distinctive properties.

Metallo-β-lactamases (MBLs) are zinc-dependent enzymes of growing clinical concern (23). They hydrolyze carbapenems, which are key antibiotics for resistant Gram-negative bacteria, and are insusceptible to serine β-lactamase inhibitors (7). Sequence information divides MBLs into three subclasses, B1, B2, and B3 (13). Subclass B2, the least studied, includes CphA (16, 20), ImiS (6, 24), and AsbM1 (25) from *Aeromonas* spp. and Sfh-I (18) from the occasional pathogen *Serratia fonticola* (4, 8, 14, 17). These are monozinc enzymes that strongly prefer carbapenem substrates. Sfh-I (18) and a class A carbapenemase, SFC-1 (12, 15), are specific to *S. fonticola* strain UTAD54. Sfh-I diverges markedly (58% ImiS and 60% CphA sequence identity) from the *Aeromonas* B2 MBLs (18). Here we expressed, purified, and biochemically characterized recombinant Sfh-I.

The complete $bla_{\text{Sfh-I}}$ gene (GenBank accession no. AF197943) (primers SfhFv4 [5′-GGATCCCATATGAATATTAAATATTTATTTACGGCTG-3′] and SfhRv2 [5′-GTGCTCGAGTTACTTAGGCGCCTTCTCAAGC-3′]) and the fragment encoding the expected mature protein (nucleotides 55 to 759; primers SfhFv3 [5′-GGATCCCATATGTCTGAAAAAAACTTAACGCTTACC-3′] and SfhRv2) were amplified by PCR; the products were ligated into vector pET-26b (Novagen), generating plasmids pSfh-Iv4 and pSfh-Iv3, respectively, and the constructs were sequenced. Protein was expressed in *Escherichia coli* BL21 Star (DE3) (Invitrogen) (37°C, M9Y medium, 50 μg/ml kanamycin, overnight induction [1 mM IPTG] at 25°C) and purified by anion exchange (Q-Sepharose; 50 mM Tris-HCl, 100 μM $ZnCl_2$, pH 8.5) and size exclusion (Superdex 75; 50 mM HEPES, pH 7.0) chromatography, yielding approximately 50 mg/liter Sfh-I at >95% purity as adjudged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Sfh-I molecular mass and metal content characteristics were investigated by electrospray mass spectrometry (QStar XL qTOF system, positive-ion mode, nanoliter/minute sample delivery, Nanomate source [0.3 lb/in^2, 1.4 kV]). The mass obtained for Sfh-Iv3 under denaturing conditions (26,136 Da; Fig. 1A) agreed with the value predicted (26,134 Da) from the sequence after removal of the N-terminal methionine. A similar result for Sfh-Iv4 confirmed removal of a 21-amino-acid signal peptide (18). Additional peaks may represent oxidized (26,136 + 16 = 26,152 Da [observed]) and subsequently hydrated (26,152 + 18 = 26,170 Da [observed]) adducts. In the native spectrum (Fig. 1B), peaks at 26,201 Da (26,136 + 65 Da), 26,218 Da (26,152 + 66 Da), and 26,239 Da (26,170 + 69 Da) indicate additions of one equivalent of zinc (65 Da) to species found under denaturing conditions. Thus, as isolated, the Sfh-I was primarily mononuclear, as observed for CphA and ImiS (6, 16) and in the Sfh-I crystal structures (Protein Data Bank [PDB] accession no. 3Q6V and 3SD9) (11), and typical of a B2 MBL.

β-Lactam hydrolysis (50 mM HEPES, pH 7.0 [25°C]) was investigated as described previously (19) (Table 1). Experiments were performed in triplicate, recording data points every

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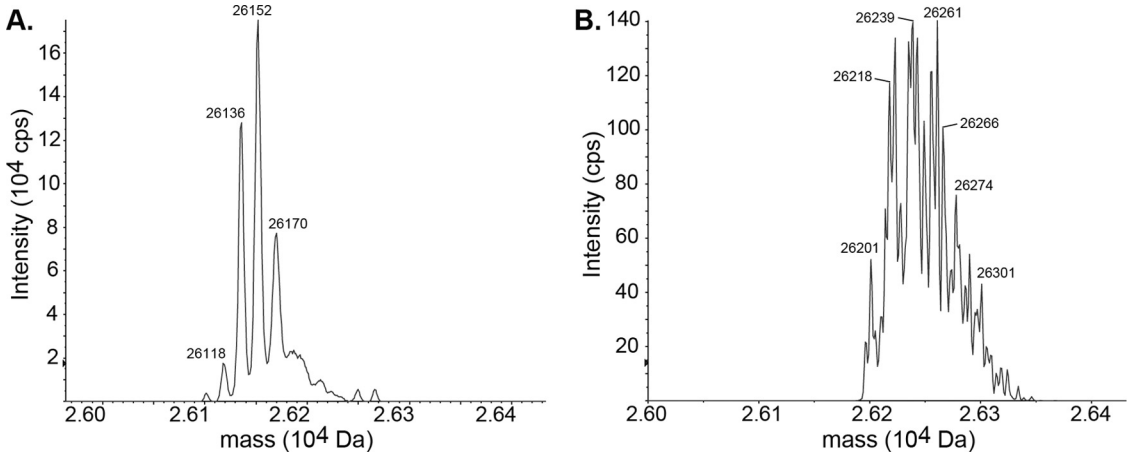

FIG. 1. Mass spectra of recombinant Sfh-I. (A) Spectrum acquired under denaturing conditions. (B) Spectrum acquired under nondenaturing (native) conditions.

3 to 4 s for 20 to 30 min and adjusting concentrations to ensure measurement of the linear phase of the reaction. Sfh-I efficiently hydrolyzes carbapenems (imipenem and meropenem $k_{cat}/K_m > 10^5$ M^{-1} s^{-1}) but resembles other B2 MBLs in its low affinity ($K_m > 300$ μM) and catalytic efficiency ($k_{cat}/K_m \leq 10^3$ M^{-1} s^{-1}) for many penicillins and cephalosporins (9, 10, 20, 22, 24, 25). This is consistent with previous data showing that Sfh-I expression in *E. coli* substantially elevated carbapenem MICs but had little effect on those for other β-lactams (18). Sfh-I hydrolyzed benzylpenicillin, cefotaxime, ceftazidime, and sulbactam, but kinetic parameters for these substrates could not be derived due to low affinities. Unexpectedly, Sfh-I (0.01 μM) hydrolyzed cefepime (against which 0.5 μM CphA shows no detectable activity [9]) with relatively high efficiency. While differences between laboratories and experimental conditions make comparison with data from previously characterized B2 MBLs difficult, we note that the low affinity of Sfh-I for penicillins is consistent with results seen with other enzymes (possibly excepting AsbM1) and that higher k_{cat} but weaker K_m values for hydrolysis of meropenem, compared to imipenem, may be a general property of subclass B2 MBLs.

CphA is inactivated by cefoxitin, moxalactam, and other cephalosporins with good 3′ leaving groups (26). Cephalosporin degradation products also inhibit the *Bacillus cereus* B1 MBL BcII (1). Whereas Sfh-I hydrolyzes cefoxitin, moxalactam, and cephalothin with low efficiency (Table 1), at higher concentrations each inhibited imipenem and nitrocefin hydrolysis. Interaction of Sfh-I with these compounds and their hydrolysis products was investigated. Although the mode of inhibition by the intact compounds was unclear, nitrocefin hydrolysis assays showed that preincubation with cephalothin or its hydrolysis product (purified by centrifugal filtration after

TABLE 1. Kinetic parameters of purified Sfh-I β-lactamase and comparison with other B2 metallo-β-lactamases[a]

Substrate	k_{cat} (s^{-1})				K_m (μM)				k_{cat}/K_m (M^{-1} s^{-1})			
	Sfh-I	CphA	AsbM1	ImiS	Sfh-I	CphA	AsbM1	ImiS	Sfh-I	CphA	AsbM1	ImiS
Ampicillin	9.5 ± 1.11	<0.01[b]	0.13	1.1	676 ± 187	2,500[b]	65	510	1.41×10^4	<4[b]	7.5×10^3	2.2×10^4
Benzylpenicillin	ND	0.03	1.2	0.53	>1,500	870	160	250	ND	35	2.0×10^3	2.1×10^3
Carbenicillin	9.15 ± 1.58	10[d]	0.44	0.5	1,280 ± 363	500[d]	28	260	7.18×10^3	2.0×10^{4d}	1.6×10^4	2.1×10^3
Nitrocefin	0.06 ± 0.003	0.0028	≤0.02	0.024[g]	106 ± 13.4	1,200	ND	51[g]	5.7×10^2	2.5	ND	4.0×10^{3g}
Cephaloridine	$8.22 \times 10^{-5} \pm 1.46 \times 10^{-5}$	<0.006	≤0.06	NH	335 ± 124.0	~6,000	ND	NH	2.46×10^{-1}	<1	ND	NH
Cephalothin	ND[h]	NR	NR	NR	84[i]	NR	NR	NR	ND	NR	NR	NR
Cefotaxime	ND	>0.0002[b]	NR	NH	>1,000	>100[b]	NR	NH	ND	2[b]	NR	NH
Ceftazidime	ND	NR	NR	NR	>500	NR	NR	NR	ND	NR	NR	NR
Cefoxitin	0.032 ± 0.00094	0.02[e]	≤0.06	NH	99 ± 7	615[e]	ND	NH	3.23×10^2	33[e]	ND	NH
Cefepime	0.64 ± 0.073	NH	NR	NR	202 ± 43.7	NH	NR	NR	3.16×10^3	NH	NR	NR
Moxalactam	0.017 ± 0.0012	NR	NR	NH	91 ± 14	NR	NR	NR	1.87×10^2	5.6[e]	NR	NH
Sulbactam	ND	0.12[c]	NR	NH	>1,000	37[c]	NR	NH	ND	3.2×10^{3c}	NR	NH
Imipenem	51 ± 2.8	1,200	71	350[f]	79.2 ± 11.2	340	230	100[f]	6.42×10^5	3.5×10^6	3.1×10^5	9.1×10^{5f}
Meropenem	109 ± 9.5	3,100	220	296[g]	215 ± 47.0	1,340	630	308[g]	5.05×10^5	2.3×10^6	3.5×10^5	3.3×10^{6g}

[a] Kinetic data displayed for CphA, AsbM1, and ImiS as reported by Vanhove et al. (22), Yang and Bush (25) and Walsh et al. (24), respectively, except where otherwise indicated. ND, values could not be determined; NH, no hydrolysis detected; NR, not reported.
[b] As reported by Bebrone et al. (2).
[c] As reported by Felici et al. (10).
[d] As reported by Segatore et al. (20).
[e] As reported by Zervosen et al. (26).
[f] As reported by Sharma et al. (21).
[g] As reported by Crawford et al. (6).
[h] Value could not be determined due to inhibition at higher concentrations.
[i] K_i value determined from inhibition of nitrocefin hydrolysis.

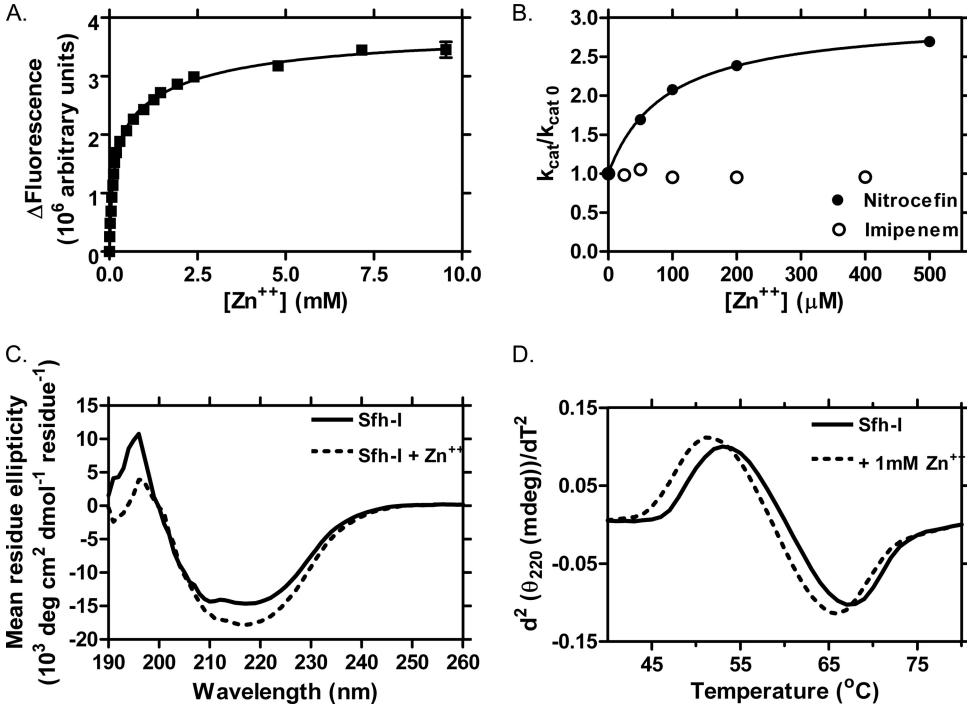

FIG. 2. Effect of additional zinc on Sfh-I. (A) Change in Sfh-I intrinsic (tryptophan) fluorescence as a function of zinc concentration (50 mM HEPES, pH 7.0; 25°C; excitation, 280 nm; emission, 341 nm). The solid line represents a fit of nitrocefin data to a two-site hyperbola model, with Y (fluorescence) = $Y_2 \times [Zn^{2+}]/(K_{d2} + [Zn^{2+}]) + Y_3 \times [Zn^{2+}]/(K_{d3} + [Zn^{2+}])$, where Y_2 and Y_3 represent the maximal fluorescence changes associated with binding of a second and third zinc equivalent, corresponding to K_{d2} (95 ± 14 μM) and K_{d3} (2,300 ± 870 μM), respectively. (B) Effect of added zinc on k_{cat}/k_{cat0} (ratio of k_{cat} at a given zinc concentration to k_{cat0} determined in the absence of excess zinc) for nitrocefin and imipenem hydrolysis. The solid line represents a fit of nitrocefin data to $k_{cat}/k_{cat0} = (K + \alpha \times [Zn^{2+}])/([Zn^{2+}] + K)$, where α represents the value of this ratio when the zinc concentration is at a saturating level (22). (C) Far-UV circular dichroism spectra (10 mM phosphate, pH 7.0; 25°C; 1-mm cuvette) of Sfh-I (8.5 μM) in the absence (solid line) and presence (dotted line) of 1 mM Zn^{2+}. (D) Second derivatives of plots of ellipticity (220 nm) against temperature for thermal unfolding of Sfh-I (8.5 μM) in the absence (solid line) and presence (dotted line) of 1 mM Zn^{2+}.

hydrolysis using IMP-1 β-lactamase) led to time-dependent inactivation. Loss of Sfh-I activity correlated with cephalothin hydrolysis, indicating that the hydrolysis product likely represents the inhibitory species. Incubating Sfh-I (3.6 μM; 18 h; 25°C; 50 mM HEPES, pH 7.0) with various hydrolysis products (1 mM) reduced hydrolysis of 200 μM imipenem by 13% (cefoxitin), 36% (cephalothin), and 94% (moxalactam). The level of activity of cephalothin-treated samples remained at 54% of the control level, even after overnight dialysis, suggesting inactivation by enzyme modification. Unfortunately, mass spectrometry failed to identify the inhibitory species; while all expected Sfh-I-derived peptides were detected, modified fragments were not, suggesting a labile inhibitory complex. Previous workers suggested that the moxalactam hydrolysis product covalently binds the CphA Cys-221 thiol (26). Association of Cys-221 with the thiol of an opened dihydrothiazine ring of the hydrolyzed cephalosporin (1), or addition to the exomethylene formed upon elimination of the 3′ leaving group, could also explain the cephalothin inactivation of Sfh-I.

CphA is most active with a single tightly bound zinc ion (K_{d1} < 20 nM) and weakly binds a second, inhibitory zinc (K_{d2} = 46 μM) (16). We used fluorescence spectroscopy (Jobin Yvon FluoroLog) to investigate the Sfh-I–zinc interaction. Titration of as-isolated (monozinc) Sfh-I with zinc (Fig. 2A) showed two distinct transitions, demonstrating binding of two further equivalents (K_{d2}, 95 ± 14 μM; K_{d3}, 2,300 ± 870 μM). The K_{d2} value is comparable to that of binding of a second zinc to CphA (46 μM [16]). The third, low-affinity zinc (millimolar K_{d3}) is unlikely to be physiologically relevant. (CphA binds a third zinc at a surface site distinct from the active site [3].)

Other B2 MBLs are maximally active as monozinc enzymes, with noncompetitive inhibition by a second zinc (6, 16, 25). The effect of zinc addition on Sfh-I activity was substrate dependent (Fig. 2B). Imipenem hydrolysis was unaffected by 400 μM zinc, while nitrocefinase activity increased due to an increased k_{cat} value. The midpoint (K) for activation (90 ± 4 μM) is equivalent to K_{d2}, indicating that a second zinc activates nitrocefinase activity. While zinc activates nitrocefin hydrolysis for some CphA mutants (2), and the ImiS M146I mutation abolishes zinc inhibition of imipenemase activity (5), the combination of zinc-dependent nitrocefinase activity and zinc-independent imipenemase activity was not previously described. Circular dichroism spectroscopy (Jasco-J810 spectropolarimeter) showed that the presence of a second zinc induced a small increase in Sfh-I secondary structure (Fig. 2C) with little effect on thermal stability (T_m for Sfh-I unfolding, 60°C; in 1 mM zinc, 58°C; Fig. 2D).

In conclusion, Sfh-I is a monozinc enzyme with a strong preference for carbapenems, confirming it to be a B2 MBL and these to be common properties of the members of this subgroup. However, Sfh-I differs from CphA, the most studied B2 MBL, in its activity toward cefepime and in the effect of addi-

tional zinc, indicating that not all B2 enzymes are inactivated by excess zinc. Further investigations should establish the basis for these differences.

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