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¹⁵N, ¹³C and ¹H backbone resonance assignments of an artificially engineered TEM-1/PSE-4 class A β -lactamase chimera and its deconvoluted mutant

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Abstract

The widespread use of β -lactam antibiotics has given rise to a dramatic increase in clinically-relevant β -lactamases. Understanding the structure/function relation in these variants is essential to better address the ever-growing incidence of antibiotic resistance. We previously reported the backbone resonance assignments of a chimeric protein constituted of segments of the class A β -lactamases TEM-1 and PSE-4 (Morin et al. in *Biomol NMR Assign* 4:127–130, 2010. doi: 10.1007/s12104-010-9227-8). That chimera, cTEM17m, held 17 amino acid substitutions relative to TEM-1 β -lactamase, resulting in a well-folded and fully functional protein with increased dynamics. Here we report the ¹H, ¹³C and ¹⁵N backbone resonance assignments of chimera cTEM-19m, which includes 19 substitutions and exhibits increased active-site perturbation, as well as one of its deconvoluted variants, as the first step in the analysis of their dynamic behaviours.

Keywords

Antibiotic resistance; TEM-1; PSE-4; β -Lactamase; Protein engineering; Chimera

Biological context

Class A β -lactamases are the most prominent group of enzymes implicated in antibiotic resistance. This class of hydrolases, comprising hundreds of members, is specific for β -

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lactam compounds such as penicillins and cephalosporins (Pimenta et al. 2014). The class A β -lactamase TEM-1 has been extensively characterized. Its structure, kinetic parameters and dynamics have been studied by X-ray crystallography, molecular dynamics simulations and NMR (Minasov et al. 2002; Savard and Gagné 2006). Its homolog, PSE-4, was also characterized by NMR and X-ray crystallography to report its structure, dynamics and kinetics (Lim et al. 2001; Morin and Gagné 2009). TEM-1 and PSE-4 share moderate sequence identity (40 %) and conserved folds (backbone RMSD 0.99 Å). Native TEM-1 and PSE-4 β -lactamases were previously recombined using the structural recombination algorithm SCHEMA to create libraries of β -lactamase chimeras, which were functionally selected for ampicillin hydrolysis (Meyer et al. 2006, 2003; Voigt et al. 2002). Because both ‘parental’ enzymes are well characterized, the artificially-evolved chimeras resulting from their recombination provide a means to characterize the effect of multiple simultaneous substitutions on enzyme structure and activity. In an effort to better understand the high tolerance of these enzymes to mutations, we previously reported the structure, function and dynamics of a highly blended TEM-1/PSE-4 chimera, cTEM-17m (Clouthier et al. 2012; Gobeil et al. 2014). This chimera harbours 17 TEM-1 to PSE-4 substitutions in one of the active-site walls (residues 150–190 according to the standard Ambler numbering scheme, i.e. numbered from 26 to 290, with gaps at positions 239 and 253 (Ambler et al. 1991) (Fig. 1). Here, we characterize a more highly perturbed chimera, cTEM-19m, sharing the same exchanged region as cTEM-17m, in addition to a second exchanged region (residues 66–73) that neighbours the catalytic Ser70 (Fig. 1). This second region introduced two additional active-site substitutions at positions 68 (M68L) and 69 (M69T). Met68 is moderately conserved in TEM-1 clinical variants while the more highly conserved Met69 has only been observed as Ile, Val and Leu variants. Chimera cTEM-19m thus holds 19 active-site substitutions relative to TEM-1 and 131 substitutions relative to PSE-4. In addition, to evaluate the impact of these two new substitutions relative to chimera cTEM-17m, we also created the deconvoluted variant of cTEM-19m in the TEM-1 context: TEM-1(M68L–M69T).

Here, we report the ^{15}N , ^{13}C and ^1H backbone NMR assignments of chimera cTEM-19m and the deconvoluted TEM-1(M68L–M69T) variant. We assigned 92.1 % of backbone amide resonances for cTEM-19m and 98.4 % for TEM-1(M68L–M69T). To verify that the missing assignments in cTEM-19m were not due to protein degradation or unfolding, we evaluated secondary structure, thermal stability and activity toward CENTA hydrolysis, confirming protein fitness.

Methods and experiments

DNA constructs, expression and purification

The original cTEM-19m gene was reported by Voigt et al. (Meyer et al. 2003; Voigt et al. 2002). Subcloning of chimera cTEM-19m into pET-24 with the OmpA signal sequence was performed as previously described (Clouthier et al. 2012). To generate the TEM-1(M68L–M69T) mutant, oligonucleotide primers were synthesized by Alpha DNA (Montréal, QC) and the QuickChange site-directed mutagenesis method developed by Stratagene (La Jolla, CA) was used with the I-Proof High Fidelity DNA polymerase (Bio-Rad, Mississauga, ON)

using pET24-TEM-1 as the template (Clouthier et al. 2012). The PCR product was DpnI-digested for 1 h at 37 °C to eliminate the methylated non-mutated parental DNA template and butanol-precipitated before being transformed into *E. coli* XL1 Blue. Cells were plated on LB-agar containing 50 µg/mL kanamycin. One colony was selected for DNA extraction to confirm the DNA sequence of the entire coding region and for retransformation into *E. coli* BL21(DE3) for expression. For NMR, uniformly [¹⁵N]- and [¹³C, ¹⁵N]-labeled cTEM-19m and TEM-1(M68L–M69T) proteins were overexpressed in modified M9 minimal medium containing ¹⁵NH₄Cl and ¹³C-glucose and purified as previously described (Clouthier et al. 2012; Gobeil et al. 2014; Morin et al. 2010). Samples were dialyzed against distilled, deionized water overnight at 4 °C. For applications other than NMR, proteins were overexpressed in auto-inducing ZYP-5052 medium (Studier 2005) and purified as previously described (Clouthier et al. 2012; Gobeil et al. 2014). Sample purity was determined by SDS-PAGE revealed by the zinc-imidazole method. Typical yields were 25 mg/L of >98 % pure protein in minimal medium and 50 mg/L in auto-inducing medium.

Solution NMR spectroscopy

[¹³C, ¹⁵N]-labeled and [¹⁵N]-labeled NMR samples were concentrated to 0.6 mM using a 10,000 MWCO Amicon concentrator. The buffer was changed to 3 mM imidazole, 0.1 % NaN₃ and 10 % D₂O, pH 6.7. The final pH was verified using a pH meter with a microprobe. All NMR experiments were performed at 31.5 °C as calibrated with a standard methanol sample (Cavanagh et al. 2006) on a Varian 18.8 T (800 MHz) NMR spectrometer equipped with a triple-resonance probe and pulsed-field gradient. ¹H–¹⁵N HSQC and a TROSY version of HNCA, HN(CO)CA, HNCACB, HN(CO)CACB and HNCO triple-resonance experiments were performed. NMR data were processed using NMRPipe (Delaglio et al. 1995) and analyzed using Sparky (Goddard and Kneller 2006) and NMRViewJ version 8.0 (Johnson and Blevins 1994).

Circular dichroism and thermal scanning fluorometry

Circular dichroism (CD) using a Jasco spectropolarimeter with a Peltier element, and thermal scanning fluorometry with Sypro Orange using a LightCycler 480 apparatus (Roche), were performed as previously described (Clouthier et al. 2012). Briefly, for CD experiments, protein solutions were at a final concentration of 0.1 mg/mL (3.5 µM) in 50 mM sodium phosphate, pH 7.0, in a 2 mm path length cuvette. Initial spectra were acquired at 25 °C from 200 to 260 nm at a scan rate of 50 nm/min. Thermal denaturation experiments were performed by monitoring changes in ellipticity at 222 nm, at a scan rate of 20 °C/h. *T_m* values were determined using the derivatives function of the Jasco Spectra Manager software with the Savitzky–Golay algorithm. For thermal scanning fluorometry, each combination of 5×, 3.33× and 2.5× SYPRO Orange solution (Invitrogen) with 4, 2 or 1 mM of test protein was prepared in 50 mM sodium phosphate, pH 7.0, to a final volume of 20 µL. Controls consisted of SYPRO Orange in buffer. Samples were heated from 20 to 95 °C with a ramp speed of 0.04 °C/s and 15 acquisitions/°C. Fluorescence was monitored with a CCD camera, using λ_{exc} = 483 nm and λ_{em} = 568 nm and a 1 s exposure time. *T_m* calculations were performed as previously described (Clouthier et al. 2012).

Kinetics

K_M and k_{cat} of CENTA hydrolysis were determined as previously reported (Clouthier et al. 2012). The hydrolysis of CENTA (35–1000 μM) was assayed with 15 nM enzyme using the extinction coefficient $\epsilon_{405\text{nm}} = 6400 \text{ M}^{-1}\text{cm}^{-1}$. The assay was performed at room temperature in 50 mM sodium phosphate, pH 7.0, using a DTX 880 plate-reader (Beckman Coulter) and 96-well plates (Corning Costar flat-bottomed model 9017) with 200 μL samples per well, giving a path length of 0.7 cm.

Assignments and data deposition

Despite their high molecular weight of 30 kDa, chimera cTEM-19m and the deconvoluted variant TEM-1(M68L–M69T) gave rise to good quality ^1H – ^{15}N HSQC spectra (Figs. 2, 3 respectively), as previously observed for TEM-1, PSE-4 and cTEM-17m (Morin et al. 2010; Morin et al. 2006; Savard et al. 2004).

Chimera cTEM-19m holds two amino acid substitutions relative to chimera cTEM-17m, originating from PSE-4 (M68L and M69T). As a result, the majority of the peaks in the cTEM-19m ^1H – ^{15}N HSQC were assigned using a superimposition strategy with the ^1H – ^{15}N HSQCs of chimera cTEM-17m (BMRB 16598) (Morin et al. 2010), TEM-1(BMR 6024, 6357 and 7238) (Savard et al. 2004), PSE-4 (BMRB: 6838) (Morin et al. 2006) and the RunAbout tool in NMRViewJ. The final level of completeness obtained for cTEM-19m was 92.1 % (232/252) of the non-proline backbone $^1\text{H}_\text{N}$ and ^{15}N resonances, 89.4 % (235/263) of $^{13}\text{C}_\alpha$ and 76.7 % (184/240) of $^{13}\text{C}_\beta$. The missing assignments in cTEM-19m (listed as supplementary material) overlap importantly with those reported for cTEM-17m and were either undetectable or gave signals too weak to allow confident assignment. Slow motions may cause peak broadening, rendering the signal indistinguishable from background noise. Most unassigned residues are clustered around the exchanged region 150–190. Active site residues D¹³¹, N¹³², A²¹⁷ and K²³⁴ were assigned in cTEM-19m though not observed in cTEM-17. Residues R²²², G²⁴⁵ and I²⁴⁶, flanking the main unassigned regions in cTEM-17m (residues 214–221, 234–244) could not be assigned in cTEM-19m, suggesting a slightly longer region experiencing slow motions relative to cTEM-17m. H²⁶ could not be identified with certitude, as it is the N-terminal residue of the mature protein and is followed by P²⁷.

Assignment of the ^1H – ^{15}N HSQC of TEM-1(M68L–M69T) variant revealed that the two mutations produced no major structural changes resonance frequency changes relative to TEM-1 indicating maintenance of the global structure. The majority of the resonances in the TEM-1(M68L–M69T) ^1H – ^{15}N HSQC were assigned using an overlaying strategy with the ^1H – ^{15}N HSQC of TEM-1 and the RunAbout tool in NMRView. The level of completeness obtained was 98.4 % (247/251) of the non-proline backbone $^1\text{H}_\text{N}$ and ^{15}N resonances, 93.5 % (246/263) of $^{13}\text{C}_\alpha$ and 87.6 % (212/242) of $^{13}\text{C}_\beta$. Missing assignments for H²⁶, S⁷⁰ and A²³⁷ were as for TEM-1 and PSE-4, resulting from peak broadening in the active-site vicinity.

The ^1H , ^{15}N and ^{13}C and backbone resonance assignments for chimera cTEM-19m and the deconvoluted variant TEM-1(M68L–M69T) have been deposited in the BioMagResBank

(<http://www.bmr.b.wisc.edu>) under the accession codes 26590 and 26586, respectively. These chemical shift assignments are the first step in the analysis of their backbone dynamic behaviour.

To verify whether the missing assignments in cTEM-19m resulted from partly unfolded or degraded protein, maintenance of its secondary structure, characterized by a high α -helical content, was confirmed by far-UV CD (Fig. 4a). The disulfide bridge between C⁷⁷ and C¹²³ was confirmed according to the C β shifts at 45.0 and 42.4 ppm, indicating oxidation. Thermal denaturation of secondary structure elements of cTEM-19m using CD at 222 nm ($T_m = 49^\circ\text{C}$; Fig. 4b) was indistinguishable from the T_m recorded by the same method for the parental TEM-1 and PSE-4 β -lactamases [49.6 and 48.6 $^\circ\text{C}$, respectively (Clouthier et al. 2012)]. The thermal stability of secondary and tertiary structure elements of both cTEM-19m and TEM-1(M68L–M69T) was determined by thermal scanning fluorometry (Fig. 4c, d). The T_m for cTEM-19m ($48.0 \pm 0.4^\circ\text{C}$) and for TEM-1(M68L–M69T) ($49.6 \pm 0.2^\circ\text{C}$) are in the same range as those reported using the same method for parental TEM-1 ($49.1 \pm 0.2^\circ\text{C}$) and PSE-4 ($47.8 \pm 1.2^\circ\text{C}$), as well as chimera cTEM-17m ($49.0 \pm 0.8^\circ\text{C}$) (Clouthier et al. 2012). These experiments confirm that cTEM-19m and TEM-1(M68L–M69T) conserve a characteristic β -lactamase-like fold, with cTEM-19m exhibiting a pattern of NMR line broadening similar to cTEM-17m.

To confirm that cTEM-19m and TEM-1(M68L–M69T) were functional, we characterized their hydrolysis of the chromogenic substrate CENTA (Table 1). Small reductions in turnover rates (k_{cat}), accompanied by a 4- to 12-fold loss of productive binding (K_M), resulted in catalytic efficiencies (k_{cat}/K_M) one order of magnitude lower than TEM-1, PSE-4 and cTEM-17m. Despite a modest reduction in activity, these results confirm that cTEM-19m and TEM-1(M68L–M69T) are well-folded, functional β -lactamase variants. Further investigations are underway to better understand the effects of these sequence substitutions on protein structure, function and dynamics.

Conclusion

¹⁵N, ¹³C and ¹H backbone resonance assignments for cTEM-19 m and the deconvoluted variant TEM-1(M68L–M69T) were achieved using double- and triple-resonance experiments. These assignments will provide the means to investigate their dynamics on a slow time scale relevant to protein function, providing insight into the effect of sequence substitutions on structure and function in β -lactamases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Relation of TEM-1 and PSE-4 to the chimeras cTEM-17m and cTEM-19m, and the deconvoluted variant TEM-1(M68L–M69T). **a** Sequence segments exchanged during chimera formation. Segments originating from TEM-1 are *blue* and from PSE-4 are *red*. The newly exchanged active-site PSE-4 segment (residues 66–73) is highlighted with *green* hatching. **b** Sequence variation introduced during chimeragenesis. Among the 17 amino acid substitutions that result from exchanging region 150–190 from TEM-1 to PSE-4 in both cTEM-17m and cTEM-19m, a Pro is exchanged for a Leu (position 174) and 2 Gly are inserted (positions 172, 175). **c** Crystal structure of cTEM-17m [PDB 4ID4; (Gobeil et al. 2014)] colored as in (a). The catalytic Ser70 is in sticks representation

Fig. 2. Two-dimensional ^1H - ^{15}N HSQC spectrum of the chimera cTEM-19m. Assigned peaks are labeled according to Ambler numbering. The section marked by a *dashed square* is presented in an expanded format. Tryptophan side chains are marked by *Wsc*. Residue 215 is marked by *plus* as its signal is of lower intensity

Fig. 3.

Two-dimensional ^1H - ^{15}N HSQC spectrum of the TEM-1(M68L-M69T) variant. Assigned peaks are labeled according to the Ambler numbering. The central section highlighted by a *dashed square* is presented in an expanded format. Wsc marks tryptophan side chains

Fig. 4.

Thermal denaturation of the chimera cTEM-19m and the deconvoluted variant TEM-1(M68L-M69T). **a** Far-UV CD spectrum at 25 °C and **b** mean molar residual ellipticity measured at 222 nm during thermal denaturation for cTEM-19m (*green*). TEM-1 (*blue*), PSE-4 (*red*) data taken from (Clouthier et al. 2012) are shown for comparison. **b** Mean molar residual ellipticity measured at 222 nm during thermal denaturation TEM-1^a (*blue*), PSE-4^a (*red*) and cTEM-19m (*green*). **c** Thermal denaturation monitored by scanning fluorimetry of cTEM-19m (*green*) and TEM-1(M68L-M69T) (*orange*) observed by fluorescence of SYPRO Orange. Melting curves are shown for a ratio of $3.33 \times$ SYPRO Orange to 1 mM (*dashed line*) or 2 mM (*full line*) protein. **d** First derivative analysis of the thermal melting curves

Table 1
Kinetic constants for the hydrolysis of the chromogenic substrate CENTA by chimera cTEM-19m and the deconvoluted variant TEM-1(M68L–M69T)

	k_{cat} (s ⁻¹)	K_M (μM)	k_{cat}/K_M (M ⁻¹ s ⁻¹)
TEM-1 ^a	33 ± 1	36 ± 5	9.2 × 10 ⁵
PSE-4 ^a	19 ± 1	46 ± 9	4.1 × 10 ⁵
cTEM-17 m ^a	48 ± 3	160 ± 35	3.1 × 10 ⁵
cTEM-19 m	11 ± 1	290 ± 43	3.8 × 10 ⁴
TEM-1(M68L–M69T)	14 ± 1	480 ± 51	3.0 × 10 ⁴

All values are reported as the average of triplicates ± standard deviation on the mean as analyzed using the Michaelis–Menten fitting in GraphPad Prism

^aValues taken from (Clouthier et al. 2012)