Maintenance of Native-like Protein Dynamics May Not Be Required for Engineering Functional Proteins

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INTRODUCTION

Our understanding of the interplay between protein dynamics and enzyme function is nascent. Beyond the large domain movements required for the function of certain proteins, we can now experimentally observe protein motions on a smaller scale with respect to amplitude and timescale. In particular, enzymes are now seen as populations of conformers in continuous exchange, some among which are conformationally tuned to bind their cognate ligand and turn it over (Fraser et al., 2009; Klinman and Kohen, 2013; Wang et al., 2012; and Whittier et al., 2013).

If enzyme dynamics are functionally relevant, cross-species conservation of dynamics may be expected. The conservation of conformational fluctuations near the active site, on the timescale of the reaction, was computationally predicted within each of the CypA, DHFR, and ribonuclease (RNase) A families (Ramanathan and Agarwal, 2011) and has been recently verified by nuclear magnetic resonance (NMR) for RNase A homologs (Gagné et al., 2012; Gagné and Doucet, 2013). In this report, we further evaluate cross-species dynamic conservation in enzymes by examining the slow timescale motions in the homologous class A TEM-1 and PSE-4 ß-lactamases. Both have been predicted by model-free calculations of NMR spin relaxation to exhibit global rigidity on the slow timescale (µs-ms). We now report their 15N Carr-Purcell-Meiboom-Gill (CPMG) NMR relaxation dispersion, experimentally confirming their conserved, high rigidity on the millisecond timescale.

Can altered protein dynamics be functionally tolerated upon enzyme modification? While slow protein motions appear to be evolutionarily conserved, is it essential to maintain native-like dynamics while engineering an enzyme? Although there has been some investigation of the impact of point mutations on dynamics and enzyme function (Bhabha et al., 2009; Fraser et al., 2009), little is known about the effects of large-scale laboratory recombination on dynamics and whether they are related to functional modifications (Doucet et al., 2009; Gagné et al., 2012). We thus investigate a laboratory-recombined chimeric TEM-1/PSE-4 ß-lactamase (Voigt et al., 2002) to explore the linkage between function and dynamics on the

SUMMARY

Proteins are dynamic systems, and understanding dynamics is critical for fully understanding protein function. Therefore, the question of whether laboratory engineering has an impact on protein dynamics is of general interest. Here, we demonstrate that two homologous, naturally evolved enzymes with high degrees of structural and functional conservation also exhibit conserved dynamics. Their similar set of slow timescale dynamics is highly restricted, consistent with evolutionary conservation of a functionally important feature. However, we also show that dynamics of a laboratory-engineered chimeric enzyme obtained by recombination of the two homologs exhibits striking difference on the millisecond timescale, despite function and high-resolution crystal structure (1.05 Å) being conserved. The laboratory-engineered chimera is thus functionally tolerant to modified dynamics on the timescale of catalytic turnover. Tolerance to dynamic variation implies that maintenance of native-like protein dynamics may not be required when engineering functional proteins.

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catalytic timescale. The TEM-1/PSE-4 chimera cTEM-17m (Morin et al., 2010) is highly related to TEM-1, although one face of its active site originates from PSE-4 (residues 150–190), introducing 17 mutations relative to TEM-1 in and near the catalytically essential Ω-loop. NMR backbone resonances, circular dichroism, and thermal scanning fluorescence assays of cTEM-17m are characteristic of a well-folded β-lactamase, and its catalytic activity is essentially indistinguishable from that of the “parental” TEM-1 and PSE-4 β-lactamases (Clouthier et al., 2012; Morin et al., 2010) (Figure 1). Nonetheless, NMR showed important backbone resonance line broadening, implying the introduction of motions on the slow µs-ms timescale in the active-site area. This suggested that laboratory recombination had a disruptive effect on protein motions (Morin et al., 2010). Model-free extraction of the S2 order parameter further suggested slow timescale motions spanning the entire cTEM-17m structure (Clouthier et al., 2012).

Here, we provide direct evidence of a far-ranging dynamic perturbation resulting from laboratory recombination. Although the high-resolution crystal structure of the chimera cTEM-17m (1.05 Å resolution) showed high structural conservation with respect to TEM-1 and PSE-4, the slow timescale dynamics sampled by 15N CPMG NMR relaxation dispersion revealed a vastly modified dynamic landscape and a dynamically perturbed active site. We demonstrate that these related class A β-lactamases have conserved rigidity on the slow timescale yet functionally tolerate broad dynamic perturbation on the timescale of catalytic turnover following laboratory engineering. These results imply that strict maintenance of native-like protein dynamics may not be required when engineering functional proteins.

RESULTS

Comparison of the Backbone Dynamics of TEM-1 and PSE-4 β-Lactamases

The class A β-lactamases TEM-1 and PSE-4 share 40% sequence identity. Their reaction mechanisms are conserved, and they recognize an overlapping set of β-lactam-type substrates. NMR spin-relaxation experiments and model-free analyses previously found TEM-1 and PSE-4 to be highly ordered on the ps-ns timescale (Morin and Gagné, 2009; Savard and Gagné, 2006). The extracted Rex exchange parameters predicted that some residues near their active sites possess slow µs-ms motions. To directly verify backbone dynamics of TEM-1 and PSE-4 β-lactamases, we performed TROSY-based 15N CPMG NMR relaxation dispersion experiments. They enable the characterization of protein motions on the ms timescale (=50–3,000 s−1), as measured by the transverse relaxation rate constant (R2) (Manley and Loria, 2012).

TEM-1 and PSE-4 β-lactamases possess few residues with significant ΔR2 (1/τex) values (Figure 2; Figure S1 and Table S1 available online). The dynamic residues in TEM-1 occur predominantly in the α/β domain, and in loop 217–225 leading into it. The global exchange rate of the four residues where the exchange rate, Kexl, could be calculated was 850 ± 210 s−1. In PSE-4, the few dynamic residues were mostly observed in the all-α domain; Kexl could be calculated only for one residue. The active-site residues Ser70 and Ala237 are the only unassigned residues of TEM-1 and PSE-4, where line broadening could be indicative of slow timescale motions (Morin and Gagné, 2009; Savard and Gagné, 2006). The lack of millisecond dynamics for the majority of residues in TEM-1 and PSE-4 is consistent with evolutionary dynamic conservation within class A β-lactamases. We also observed elevated R2 at a number of homologous residues in TEM-1 and PSE-4, including the active-site area residues Ser71, Val216, and Gly238 (Figure S2). This implies that those residues undergo molecular motions faster than the upper limit probed by CPMG (=3,000 s−1) (Kempf and Loria, 2004) and suggests that some among these restricted conserved molecular motions may be functionally relevant.
High-Resolution Structure Determination of the Chimeric β-Lactamase cTEM-17m

It is important to determine how protein engineering alters dynamics: if modified dynamics alter aspects of function, this could modulate our capacity to engineer functional proteins. Because protein recombination blends segments of homologous proteins and can minimize the disruption of intraprotein contacts (as for the SCHEMA recombination algorithm (Voigt et al., 2002)), it is reasonable to assume that functional chimeric proteins should conserve structural and, potentially, dynamic homology with their “parental” proteins. To verify this hypothesis, the high-resolution structure and the slow timescale dynamics of the TEM-1/PSE-4 β-lactamase chimera cTEM-17m were examined. High-quality crystals of cTEM-17m were obtained. Molecular replacement and further refinement following X-ray diffraction analysis resulted in a high-resolution structure model (1.05 Å resolution; Protein Data Bank [PDB] ID: 4ID4; Figure 3A; Table S2). The α-carbon root-mean-square deviation (RMSD) was smaller with respect to TEM-1 than PSE-4 (Table 1; Figure 3B), consistent with their respective sequence divergences. Structural comparison using the secondary structure matching (SSM) function implemented in COOT showed a similar trend (Table 1). This confirms maintenance of a TEM-1-like structure, despite the 17 residues exchanged with PSE-4. The high-resolution structure revealed higher anisotropy on the surface of the α/β domain as well as on the side chain of Tyr105. However, observation of anisotropy in an individual structure should not be interpreted as necessarily indicating motion, because one cannot discriminate between inherent disorder and artifacts due to the crystalization process.

Exchanged Region Constituting the Ω-Loop

The active-site region of cTEM-17m is chimeric (Figure 1): the catalytically important Ω-loop (residues 161–179) originates from PSE-4, within a TEM-1-like active site. The 17 substitutions...
in the exchanged segment 150–190 produced no major structural changes in the Ω-loop area, despite the introduction of two negatively (A150D, V159E) and two positively (I173K, A184K) charged residues with no counterbalancing loss of charge (Figure S3). We observed only a 1 Å displacement of the backbone of 174–177, located on the protein surface, relative to TEM-1 and PSE-4. This region includes the exchanged residues I173K, P174L, and N175G. Modification of the central residue of the Ω-loop (E168D) did not visibly alter its interactions within the protein.

**Greater Active-Site Area and Active-Site Walls**

Despite high structural conservation in the exchanged region 150–190, significant differences were observed in the greater active-site area of cTEM-17m. Strikingly, the volume of its active-site cavity (746 Å³) was estimated to be nearly twice that of TEM-1 (389 Å³), mainly as a result of the reorientation of the Tyr105 side chain and of loop 213–220. The Tyr105 phenol ring cTEM-17m was assigned to the m-30° conformation (Figures 3C and 3D), in contrast with the flipped t80° conformation assigned in all native and mutant apoenzyme structures of TEM-1 and PSE-4 (Table S3). We note that its side chain showed the highest anisotropy. The predominant m-30° conformation observed here may be a consequence of the dynamics of Tyr105 rather than of a structural alteration relative to TEM-1; further investigation of the motion of this “gatekeeping residue” is ongoing.

The active-site wall formed by the random coil loop 213–220 connects the all-α domain and the α/β domain and lies across the active site from the exchanged sequence block, which it does not contact. Residues 214–217 were significantly displaced in cTEM-17m relative to TEM-1 and PSE-4 (Figure 3D). The Val216 backbone carbonyl and the Arg244 guanidinium stabilize a conserved water molecule, which then stabilizes the sub-

**Comparison of NMR Chemical Shift Assignments**

Comparison of the reported backbone chemical shift assignments of cTEM-17m (Morin et al., 2010) with TEM-1 (Savard et al., 2004) immediately suggests that cTEM-17m has altered slow-timescale dynamics. In TEM-1, all residues were assigned except for the catalytic nucleophile, Ser70, and Ala237. In a striking contrast, despite producing spectra of comparable quality (Figure S4), backbone resonance assignments could not be ascribed to 8.8% of cTEM-17m (22 of 251 assignable residues; Table S1) due to exchange broadening (Figure 4). Important regions of cTEM-17m undergo slow conformational exchange, indicative of altered dynamics. Notably, backbone 1HN and 15N resonance assignments were missing for the active-site residues Asp131 of the SDN loop (Ser130, Asp131, Asn132); the majority of the loop 213–220 interdomain linker region implicated in substrate positioning (Huang et al., 1996; Sabbagh et al., 1998), cTEM-17m is functionally tolerant of the enlarged active-site conformation.

**Figure 3. Crystal Structure of cTEM-17m Overlaid on TEM-1**

(A) Backbone overlay of the crystal structures of cTEM-17m (PDB ID: 4ID4, yellow) and TEM-1 (PDB ID: 1ZG4, blue).

(B) Cα RMSD per residue of cTEM-17m relative to TEM-1 (PDB ID: 1ZG4; blue) and PSE-4 (PDB ID: 1G68; red).

(C) Overlay of the active-site residues in cTEM-17m and TEM-1 structures.

(D) Overlay of loop 214–217 including side chains of cTEM-17m and TEM-1. Key residues of the active site of (S70, Y105, and S130) are shown as a reference for orientation. The main-chain RMSD for residues 214–217 is 1.93 Å relative to TEM-1 (4.05 Å for all atoms), and 2.04 Å relative to PSE-4 (2.95 Å for all atoms). The conserved water molecule stabilizing benzylpenicillin (B2) in BZ-bound TEM-1 (Wat294; PDB ID: 1FQG) overlays with Wat1001 in apo-TEM-1 (blue sphere) and Wat431 in cTEM-17m (yellow sphere). See also Table S2.
cTEM-17m on a slow timescale. We note that the region comprising the residue substitutions themselves (150–190) was well resolved.

Significant chemical shift differences ($\Delta\delta$) were mapped relative to TEM-1 (Figure 5A). As a direct result of the amino acid substitutions within the exchanged segment 150–190, modified short-range interactions were observed, particularly for the conserved Ala185 and Ala187 sandwiched between substituted residues. Longer range perturbations were also observed, such as the 0.4–0.5 parts per million (ppm) $\Delta\delta$ of residues 63–65. They are at the heart of the active-site pocket and lie within 5.5 Å of the M182T substitution, suggesting that those variations are related (Figure 5B). Residues Thr118, Thr140, Arg222, and Ala280, located as far as 26 Å from the exchanged region 150–190, also exhibited significant $\Delta\delta$. Displacement of the active-site wall 213–220, discussed earlier, has modified the network extending through Arg222 and Ser223 to Leu220 and Ala280 (Figure 5C) as reflected in the high $\Delta\delta$ of Arg222 and Ala280; Leu220 was unassigned.

### Table 1. Comparison of the Crystal Structure of Chimera cTEM-17m with Its Parental Homologs TEM-1 and PSE-4

<table>
<thead>
<tr>
<th>Homolog</th>
<th>PDB ID</th>
<th>Resolution (Å)</th>
<th>RMSD$^a$ with cTEM-17m (or TEM-1) (Å)</th>
<th>SSM RMSD$^b$ with cTEM-17m (or TEM-1) (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM-1 (Stec et al., 2005)</td>
<td>1ZG4$^c$</td>
<td>1.55</td>
<td>0.70</td>
<td>0.53</td>
</tr>
<tr>
<td>PSE-4 (Lim et al., 2001)</td>
<td>1G68</td>
<td>1.95</td>
<td>1.38 (1.25)</td>
<td>1.15 (0.98)</td>
</tr>
</tbody>
</table>

$^a$-carbon RMSD.

$^b$Secondary structure matching (SSM) RMSD function implemented in COOT.

$^c$TEM-1 (PDB ID 1ZG4) was selected for comparison to cTEM-17m (1.05 Å resolution; PDB ID 4ID4) due to the absence of SO$_4$ in both active sites.

Chimera cTEM-17m Exhibits Increased $^{15}$N Millisecond Backbone Dynamics

The missing backbone assignments in cTEM-17m, indicative of increased millisecond timescale motions, are consistent with the inference of increased slow motions from the model-free formalism (Clouthier et al., 2012; Morin and Gagné, 2009; Savard and Gagné, 2006). Crystal structure (as discussed in the present study), circular dichroism, and thermal scanning fluorescence (Clouthier et al., 2012) are all consistent with a well-folded protein, excluding local unfolding or aggregation as being the cause of the increased line widths. Backbone $^{15}$N CPMG relaxation dispersion for cTEM-17m was performed to experimentally verify the dynamic effects of substituting multiple residues in the active-site area. The relaxation dispersion data revealed 60 assigned residues which undergo conformational exchange with $\Delta R_2 (1/T_{1p}) > 7.0 \text{ s}^{-1}$ (Figure S6 and Table S1) and were therefore deemed to undergo slow timescale motion. This differs importantly from TEM-1 and PSE-4 (11 and 5 residues, respectively), and directly confirms that the chimeric cTEM-17m has strikingly increased conformational exchange on the timescale of $\approx 50–3,000 \text{ s}^{-1}$ (Figure 2). Repeating the experiment with a 3-fold diluted sample confirmed that the dispersion profiles observed were not due to aggregation. Residues exhibiting conformational exchange are located throughout the protein, with many clustering in the active-site area and near the N- and C-terminal $\alpha$ helices. While only two of the 17 substituted residues of cTEM-17m (Gly172 and Lys173) were found to be dynamic on that timescale, several conserved residues in the 150–190 segment showed increased conformational exchange: the catalytically relevant Glu166 and Asn170, as well as Leu169 and Glu171. The active-site residues Met69, Thr71, Phe72, Met129, Asn132, Tyr105, and Ser106 also showed altered motions in cTEM-17m. Thus, the substitutions in the exchanged segment 150–190 have dramatically altered the slow dynamics in the active-site area and throughout the enzyme.

![Figure 4. Chimera cTEM-17m Has Altered Slow-Timescale Dynamics](image-url)

Zoomed views for residues N132, A217, G218, L221, and R244 exhibiting line broadening in cTEM-17m, consistent with increased slow-timescale dynamics at positions relative to wild-type TEM-1.
Active-Site Dynamics Do Not Hinder Engineering

We note that, in TEM-1, PSE-4, and cTEM-17m, the mean transverse relaxation rate was in the range of 10 s⁻¹, indicating a similar extent to which µs-ms motions were not suppressed. A similar number of residues exhibit transverse relaxation rates above the SD for the mean R² (29 residues in TEM-1, 25 in PSE-4, and 33 in cTEM-17m) (Figure S2).

DISCUSSION

Over 200 natural variants of TEM-1 (http://www.lahey.org/Studies/temtable.asp) procure resistance to β-lactam antibiotics. Laboratory evolution has examined the high mutability of β-lactamases (Deng et al., 2012), including simultaneous mutations and concurrent recombinations which are less likely in nature (De Wals et al., 2009; Meyer et al., 2003). This has revealed that class A β-lactamases are highly adaptable, readily maintaining activity and broadening substrate specificity. TEM-1 variants of the active-site Tyr105 showed a correlation between inferred slow dynamics and catalytic efficiency, suggesting that motions promote function in class A β-lactamases (Doucet et al., 2007). The evolvability of TEM-1 was found to include a weak functional tradeoff with respect to latent and promiscuous protein function (Soskine and Tawfik, 2010). This tradeoff may result from protein conformational flexibility, where a population of alternative conformations would mediate the new function without severely compromising the conformation mediating the native function. In other words, tolerance to modifications in dynamics may be one of the mechanisms for sustaining evolutionary variability.

Here, a system comprising both natural and laboratory-engineered homologs of β-lactamases provides further insight into the role of dynamics in enzyme function. We experimentally observed the conservation of high rigidity in TEM-1 and PSE-4 β-lactamases on the slow timescale, accompanied by millisecond timescale motions in a restricted number of residues. The inclusion of slow motions at the active site supports the hypothesis that they play a functional role and that they may be evolutionary conserved (Gagné et al., 2012; Ramanathan and Agarwal, 2011). The slow motions previously predicted by Lipari-Szabo model-free analysis were only partially confirmed: model-free formalism overestimated the number of residues possessing dynamic character on the slow timescale (≈50–3,000 s⁻¹), predicting 20 and 32 residues undergoing slow dynamics in TEM-1 and PSE-4, respectively (Morin and Gagné, 2009; Savard and Gagné, 2006), while 11 in TEM-1 and 5 in PSE-4 were experimentally identified by 15N CPMG relaxation dispersion. Molecular dynamics simulations and model-free analysis of TEM-1 and PSE-4 are in agreement with their being highly ordered. The high-amplitude motions that were computationally predicted at the tip of the Ö-loop (Gös and Pleiss, 2009; Fisette et al., 2010; Morin and Gagné, 2009) were not observed here, potentially because the timescales examined differed.

The structure-based recombination of TEM-1 and PSE-4 generated chimera cTEM-17m, which has a catalytic efficiency (catalytic rate constant/Michaelis-Menten constant; kcat/Km) similar to the parental TEM-1 and PSE-4 toward five β-lactam substrates (Figure 1C). This demonstrates that the catalytic machinery of cTEM-17m is intact (similar kcat), though some alterations in productive substrate binding have occurred (some increases in Km) (Clouthier et al., 2012). The high-resolution crystal structure confirms that the laboratory-generated cTEM-17m is only subtly altered, despite the poor tolerance to mutation of several residues in the exchanged 150–190 segment (Deng et al., 2012; Huang et al., 1996). However, cTEM-17m exhibits a vastly modified dynamic pattern on the slow timescale relative to both parental enzymes. The extent of dynamics observed by 15N CPMG (60 residues with ΔR² (1/T2) ≥ 7.0 s⁻¹) was consistent with model-free analysis (61 residues predicted) (Clouthier et al., 2012). While the predicted residues differed significantly, they generally clustered in the same regions, suggesting that the timescales sampled by model-free analysis and by 15N CPMG differed to some extent.

Some of the 60 residues showing significant relaxation dispersion in cTEM-17m have structural or functional relevance. Simulated annealing of TEM-1 previously revealed conformational...
exchange between m and t rotamers of Tyr105, which may favor ligand binding (Doucet et al., 2004; Doucet and Pelletier, 2007). Here, the m rotamer was captured in an apoenzyme structure (Figure 3C). Conformational exchange for Tyr105 was observed by 15N CPMG only in cTEM-17m (Figure 6A), indicating that the timescale of the conformational exchange in TEM-1 must be faster or slower than that investigated. The global exchange rate for Tyr105 and its neighbor Ser106 was 300 ± 90 s⁻¹/C0, similar to other active-site regions such as the environment of Ser70 (Met69 and Phe72, global kex = 480 ± 110 s⁻¹), Glu166 (kex = 330 ± 90 s⁻¹), and the S130 region (Thr128 and Met129, kex = 630 ± 100 s⁻¹) (Figures 2D and 6B). The global exchange rate for those active site regions was 440 ± 70 s⁻¹; we note the coincidence with kcat (benzylpenicillin) = 480 s⁻¹. For TEM-1, the rate-limiting step for hydrolysis of penicillins is deacylation, while hydrolysis of cephalosporins (first and third generation) is rate limited at the acylation step (Saves et al., 1995). There is no evidence pointing to a change in rate-limiting step for cTEM-17m. As the slow dynamics of cTEM-17m differ from those of TEM-1, acylation (as verified by cephalosporin hydrolysis) and deacylation (as verified by penicillin hydrolysis) are shown to tolerate altered dynamics. In particular, the native-like catalytic efficiency of cTEM-17m indicates that the U-loop residue Glu166, which appears to be implicated in the deacylation step (Saves et al., 1995) can be rigid (TEM-1 and PSE-4) or more dynamic (cTEM-17m) on the timescale of catalytic turnover (Figure 6). Thus, conservation of slow motions is not essential for the rate-limiting acylation and deacylation steps.

Figure 6. 15N CPMG Conformational Dynamics for Residues Selected on the Basis of Altered Dynamics in cTEM-17m
(A) 15N CPMG relaxation dispersion curves for active-site residues Tyr105 and Glu166; oxyanion hole residues Asp233 and Ile247 (unassigned region 234–244 in cTEM-17m), and helix h1, Lys32 and h11, Ile279 for TEM-1 (blue), PSE-4 (red), and cTEM-17m (black). All relaxation dispersion curves are shown in Figure S6. The exchange rate, kex, for cTEM-17m was obtained from two-field fits of 15N CPMG data acquired at 800 MHz (full line) and 600 MHz (dashed line).
(B and C) The catalytic Ser70 is presented in sticks. The active site is indicated by the benzylpenicillin (BZ) substrate (from PDB ID 1FQG, overlaid on the cTEM-17m structure PDB ID 4ID4) and the catalytic Ser70, both in green sticks. Unassigned residues are dark gray. Residues with a D2R (1/t2cp) greater than 7 s⁻¹/C0 are colored in yellow to red gradient as in Figure 2D. (B) Residues Tyr105, Glu166, Asp233, and Ile247 are in sticks. (C) Lys32 and Ile279 of the N- and C-terminal helices h1 and h11 are in sticks. The figures were generated using PyMOL. See also Figure S6.
Indirect evidence of increased slow timescale dynamics of cTEM-17m suggested by the incomplete backbone assignment is now supported by NMR observation of increased dynamics flanking the unassigned residues. Residues 231–233 and 247–248 (global $k_{ex} = 1,100 \pm 250\ s^{-1}$), flanking the unassigned 234–244 segment (Figure 6), exhibit increased slow dynamics, confirming the introduction of slow conformational exchange in this region. This shift in motion is striking, given that this region participates in the oxyanion hole and is linked to substrate recognition (Marciano et al., 2009).

The N- and C-terminal helices (h1 and h11) pack side by side and on to a five-stranded antiparallel $\beta$ sheet to constitute the $\alpha/\beta$ domain. Chimera cTEM-17m showed significantly increased slow timescale dynamics in both terminal helices, despite being distal from the exchanged sequence block 150–190 and from the active site (Figures 6A and 6C). High crystallographic B-factors (Val31, Lys32, Gln39, and Glu281) and residues identified as having double occupancy (Asp35, Asp38, Glu274, and Arg275) also suggest motion. During functional selection of the libraries of $\beta$-lactamase chimeras, having both terminal helices (h1 and h11) originating from the same parent was correlated with a greater selective advantage (Meyer et al., 2006). The exchange rate, $k_{ex}$, is matched for both helices (450 ± 60 $s^{-1}$ and 440 ± 50 $s^{-1}$ for h1 and h11, respectively), suggesting that the altered, correlated motions in the cTEM-17m terminal helices maintain integrity of the $\alpha/\beta$ domain.

The active-site cavity of cTEM-17m was found to be significantly enlarged as a result of displacement of potentially mobile elements: Tyr105 and loop 213–220. Enlargement of the cavity has been proposed to favor hydrolysis of bulky third-generation cephalosporins among some extended-spectrum $\beta$-lactamases (ESBLs) (Chen et al., 2008), while others maintain a cavity size similar to TEM-1 but show anisotropic B-factors in the $\beta$-strands (residues 230–237) (Delmas et al., 2008), suggesting motion, which may play a functional role (Chen et al., 2005). The $\beta$-strand was unassigned in cTEM-17m, suggesting that it has adopted motions similar to those that evolved naturally in the CTX-M ESBLs. Although the $\beta$-strand of cTEM-17m did not exhibit higher than average B-factors, neighboring residues 227–229 did (Figure S4); motions inferred from NMR in solution were apparently frozen out in the crystal form. Despite increased active-site cavity size and dynamic changes observed or inferred in residues 233–247, chimera cTEM-17m has native-like hydrolytic capacity toward CTX (Clouthier et al., 2012); we note that this phenotype had not been selected for during laboratory evolution (Voigt et al., 2002). Our results indicate that the active-site area functionally tolerates variations in dynamics, which may contribute to the evolvability of class A $\beta$-lactamases.

Our results are consistent with the $\beta$-lactam hydrolase function of class A $\beta$-lactamases (native TEM-1 and PSE-4) having naturally evolved within a rigid framework supplemented with a limited number of motions on the catalytic timescale yet also demonstrate that the fold and hydrolytic function are compatible with a drastically altered dynamic pattern (laboratory-evolved cTEM-17m). This work vividly illustrates the adaptability of class A $\beta$-lactamases to variations in dynamics on the timescale of catalysis. The conservation of slow dynamics in naturally evolved enzymes (in addition to the present article: Gagné et al., 2012; Ramanathan and Agarwal, 2011) is not at odds with variation of dynamics following laboratory engineering and functional selection. Laboratory evolution does not limit a natural setting where nutrient scarcity, rigors of life as a single-cell organism, and lower expression levels may reveal detrimental aspects of the increased dynamic makeup that were not identified here. Furthermore, some evidence suggests that the motions observed in cTEM-17m may result from a shift in timescale: residues 212, 215, 216, and 221 in TEM-1 exhibit elevated transverse relaxation rate (Figure S2), while residues 217–218 exhibit millisecond dynamics (Figure S1). This region (214–221) is mostly unassigned in cTEM-17m, suggesting that motions in the two proteins occur on a different timescale. Thus, it may be that underlying dynamics in TEM-1 and PSE-4 are not probed by the current $^{13}$N CPMG experiments. Proteins exist as a statistical ensemble of conformations where the native state is the predominantly accessible state (Boehr et al., 2010; Henzler-Wildman et al., 2007). Conformational heterogeneity is increasingly being linked to protein function (Bhattacharya et al., 2011; Boehr et al., 2009). Mutations may shift the conformational equilibrium, making alternative conformers more accessible and permitting promiscuous functions and specificities (Boehr et al., 2009). A putative underlying dynamic population in the parental TEM-1 and PSE-4 could contribute to the evolvability of $\beta$-lactamases. Indeed, the high evolvability of $\beta$-lactamases is seen in their divergent natural evolution to numerous homologs and in their “real-time” evolution as evidenced by the hundreds of drug-resistant clinical isolates with altered specificity (Bush, 2013). The $\Omega$-loop interchange in cTEM-17m may have unveiled an underlying dynamic population. The observed fitness of chimera cTEM-17m suggests that tolerance to variations in the dynamic landscape may be an enabling factor in natural evolution: if not absolutely fixed in evolution, a modified dynamic landscape could provide access to new evolutionary paths.

The concept of enzyme dynamics being linked to evolvability has only recently been proposed as one of four criteria associated with the potential evolvability of an enzyme toward a promiscuous function (Glembo et al., 2012; Tokunji and Tawfik, 2009) and, in particular, with respect to evolvability of class A $\beta$-lactamases (Risso et al., 2013). More generally, the alteration of an enzyme’s dynamic makeup may shift the distribution of available states in solution to favor one or more possessing a new binding preference. The present relaxation dispersion study suggests that class A $\beta$-lactamases possess the potential for inherent flexibility within the active site and throughout the structure, in the setting of a rigid native state. Given the requirement for $\beta$-lactamases to evolve rapidly, we propose that the high evolvability of their broad substrate promiscuity is linked with a tolerance to dynamic substrate population shifts.

**SIGNIFICANCE**

This work examines the relation between protein dynamics (or motions) and function in both native and engineered homologs of an enzyme. First, we determined that the motions on the catalytic timescale for two homologous $\beta$-lactamases are few but that some occur within the active site. This supports the hypothesis that, like sequence and structure, millisecond protein motions are conserved throughout natural evolution. A growing body of evidence indicates that
motions on the slow timescale are implicated in enzyme function. This could have important implications for the field of protein engineering if proteins cannot withstand alterations in their dynamics that would occur on modifying the amino acid sequence. We thus verified the slow motions of a laboratory-engineered chimera, which had been previously produced by blending fragments of those homologous β-lactamasases. While the chimera was fully functional and its structure essentially unchanged, its slow motions were vastly altered. This illustrates that an equally efficient enzyme can be engineered, which differs from its natural homologs by its dynamic properties. In effect, a tolerance to dynamic alterations should render proteins more evolvable than an incapacity to adapt to motional alterations.

**EXPERIMENTAL PROCEDURES**

**Materials**
Nitrocefin was purchased from Calbiochem. Kanamycin, isopropyl-1-thio-β-D-galactopyranoside, components for growth media, and purification materials were from BioShop Canada. 3H-NH₄Cl and 2H₂O were purchased from Cambridge Isotope Laboratories. Tris-Cl, polyethylene glycol (PEG) 4000, and MgCl₂ used for protein crystallization were from Sigma-Aldrich.

**Protein Expression and Purification**
The cTEM-17m expression construct has been described elsewhere (Morin et al., 2010). Expression was essentially as performed elsewhere (Doucet et al., 2007; Morin et al., 2010; Savard and Gagné, 2006). For crystallography, a 4 ml overnight culture in ZYP-0.8% auxoinducing media (Studier, 2005) was used to inoculate 400 ml of ZYP-5052 containing 30 μg/ml kanamycin. Cells were propagated with agitation at 37°C for 2 hr, then overnight at 22°C. Induced cells were sedimented by centrifugation (5,000 × g, 30 min, 4°C). The cell pellet was resuspended in 50 ml of 10 mM Tris-Cl, pH 7.0. Lysis was performed using a Constant Systems cell disruptor. Cellular debris were sedimented by centrifugation (20,000 × g, 30 min, 4°C), the supernatant was filtered through a 0.2μm filter, and the protein was purified as described elsewhere (Doucet et al., 2007; Morin et al., 2010; Savard and Gagné, 2006), with the following modifications. Following sample application, the DEAE-Sepharose Fast Flow column (1.6 cm × 30 cm) was washed with 3 column volumes (CV) of 10 mM Tris-Cl, pH 7.0. A linear gradient to 200 mM Tris-Cl, pH 7.0, was achieved over 4 CV, and the column wash was achieved over 3 CV. Fractions containing β-lactamase were identified using a nitrocefin hydrolysis assay (Calbiochem) and by 15% SDS-PAGE with Coomassie blue staining. Fractions containing ≥75% β-lactamase were concentrated to 1.5 ml using an Amicon concentrator (MWCO 10000, Millipore) and applied to a Superdex 75 column (1.6 cm × 55 cm) pre-equilibrated with 50 mM Tris-Cl, pH 7.0. Elution was at a flow rate of 1 ml/min. Fractions containing β-lactamase were identified by 15% SDS-PAGE with zinc-imidazole staining. Sample purity was ≥95% as estimated on gel with ImageJ (http://rsb.info.nih.gov/ij/). Enzyme concentration was determined with the Bradford assay (Bio-Rad). For NMR experiments, unlabeled and uniformly [15N]- and [1H, 15N]-labeled TEM-1, PSE-4, and cTEM-17m samples were overexpressed in modified M9 minimal media containing 15NH₄Cl and 100% 2H₂O and purified as described elsewhere (Doucet et al., 2007; Morin et al., 2010; Savard and Gagné, 2006). Samples were dialyzed against distilled, deionized water overnight at 4°C and concentrated to 0.6 mM using a 10000 MWCO Amicon concentrator while the buffer was changed to 3 mM imidazole, 0.01% NaN₃, and 10% D₂O, pH 6.7. The final pH was verified using a pH meter with a microprobe.

**Crytalization of cTEM-17m and Data Collection**
cTEM-17m was concentrated to 25 mg/ml (0.8 mM) in 50 mM Tris-Cl, pH 7.0. Crystals were grown at 22°C in hanging drops prepared by mixing 1 μl of the protein solution and 1 μl of the reservoir solution (100 mM Tris-Cl, pH 8.0, 26% PEG 4000, and 0.25 M MgCl₂). Diffraction data were collected from a single crystal flash-frozen in liquid nitrogen, at the Canadian Macromolecular Crystallography Facility Beamline 08ID-1 (Canadian Light Source). Data processing and refinement statistics are summarized in Table S2. The collected diffraction images were processed with the XDS program package (Kabsch, 2010). The initial structure model was constructed by molecular replacement with Phaser (McCoy et al., 2007), using a native TEM-1 structure (PDB ID: 1ZG4) (Stec et al., 2005) as an input model. The structure model was further improved through iterative rounds of manual model building with COOT (Emsley and Cowtan, 2004) and automated refinement with PHENIX (Adams et al., 2010) and REFMACS (Vagin et al., 2004). Data collection and refinement statistics are summarized in Table S2.

**Solution NMR Experiments**
All NMR experiments were performed at 31.5°C as calibrated with a standard methanol sample (Cavanagh et al., 2007) on Agilent 18.8 Tesla (18.8T) and 11.7T TEM-1 and PSE-4 or 14.1T (cTEM-17m) NMR instruments equipped with a triple-resonance probe and pulse-field gradient. [1H, 15N]-labeled TEM-1, PSE-4, or cTEM-17m (0.6 mM) were used to perform all relaxation experiments. Backbone resonances for TEM-1, PSE-4, or cTEM-17m were previously assigned (TEM-1 Biological Magnetic Resonance Bank [BMRB] IDs: 6024, 6357, and 7328; PSE-4 BMRB ID: 6838; cTEM-17m BMRB ID: 16598) (Morin et al., 2006; Savard and Gagné, 2006), μ-s ms protein dynamics for TEM-1, PSE-4, and cTEM-17m were characterized using 1H TROSY relaxation-compensated CPMG (rCPMG) experiments (Loria et al., 1999). NMR relaxation data was typically acquired using a spectral width of 13,000 Hz with the 1H carrier frequency centered on the water resonance. The 15N carrier frequency was centered at 120 ppm with 15N spectral widths of 2400, 2100, and 2800 Hz for TEM-1, PSE-4, and cTEM-17m, respectively. Sixteen transients were acquired for each of 128 t₁ points on the 18.8T and doubled for the 11.7 and 14.1T magnets. Spin-relaxation rate constants at each CPMG pulse repetition time (tₚ) were acquired in a constant time manner. Transverse spin-relaxation data using the TROSY rCPMG experiment for TEM-1, PSE-4, and cTEM-17m were acquired with interpulse delays, tₚ, of 0.0, 0.625, 0.714 (x2), 1.0, 1.25, 1.67, 2.0, 2.50, 3.33, 5.0, and 10.0 ms during the nitrogen relaxation period for a constant, total relaxation time of 20 ms. The individual spectra were acquired as an interleaved 3D experiment in which the 2D planes were extracted and peak intensities determined from a 3 x 3 grid using software written and provided by J. Patrick Loria et al. (Yale University). Conformational exchange was considered when the difference in measured R₂ [ΔR₂ (1/tₚ)] at fast (tₚ = 0.625 ms) and slow (tₚ = 10 ms) pulsing rates on the 18.8T data set was greater than 7 s⁻¹ in relaxation dispersion profiles. This threshold was selected based on relaxation dispersion error estimation to eliminate false-positives resulting from a poor fit. To verify that the dispersion curves obtained for cTEM-17m were not a result of aggregation, the 15N CPMG experiment was performed on a diluted (0.2 mM) cTEM-17m sample in identical buffer conditions at 14.1T. Sensitivity was increased by increasing the scan number to 64 to compensate for low sample concentration.

**ACCESSION NUMBERS**
The structure of cTEM-17m was deposited to the PDB under the ID code 4ID4.

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes six figures and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2014.07.016.

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We thank Michelle M. Meyer and Frances H. Arnold for providing the cTEM-17m construct; Tara Sprules and Sameer Wahid of the Québec/Eastern
Canada High Field NMR Facility for technical assistance; Michael Osborne of the Institute for Research in Immunology and Cancer Biophysics Research Facilities; and J. Patrick Loria for insight into relaxation parameters. This work was supported by National Sciences and Engineering Research Council of Canada Discovery Grants Program—Individual #227853 (to N.D.) and #402623 (to N.D.) and by Canadian Institutes of Health Research Grant MOP-131070 (to A.M.B.). A.M.B. holds a Canada Research Chair in Structural Biology, and N.D. holds a Fonds de la Recherche en Santé Québec (FRQS) Research Scholar Junior 1 Career Award. S.G. is the recipient of a FRQNT Graduate Scholarship. J.P. is supported through a FRQoS postdoctoral fellowship, and D.G. holds an Alexander Graham Bell Canada Graduate Scholarship.

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REFERENCES


Supplemental Information

Maintenance of Native-like Protein Dynamics May Not Be Required for Engineering Functional Proteins

Sophie M.C. Gobeil, Christopher M. Clouthier, Jaeok Park, Donald Gagné, Albert M. Berghuis, Nicolas Doucet, and Joelle N. Pelletier
SUPPLEMENTAL TABLES

Table S1, related to Figure 2: \( \Delta R^2 \) values \( \geq 7 \) s\(^{-1} \), derived from TROSY-based \(^{15}\)N-CPMG experiments, and unassigned residues (NA) in TEM-1, PSE-4 and cTEM-17m.

(see accompanying Excel spreadsheet)

Table S2, related to Figure 3: Data collection and refinement statistics for the crystal structure of the cTEM-17m chimera (PDB 4ID4).

<table>
<thead>
<tr>
<th>Data collection</th>
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<tbody>
<tr>
<td>Space group</td>
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<tr>
<td>Unit-cell parameters (Å)</td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>36.73</td>
</tr>
<tr>
<td>b</td>
<td>58.72</td>
</tr>
<tr>
<td>c</td>
<td>109.3</td>
</tr>
<tr>
<td>Resolution (Å)</td>
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<tr>
<td>Completeness (%)</td>
<td>95.8 (92.6)</td>
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<tr>
<td>Redundancy</td>
<td>7.3 (7.4)</td>
</tr>
<tr>
<td>I/(\sigma(I))</td>
<td>27.3 (3.9)</td>
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<tr>
<td>R-merge</td>
<td>0.046 (0.461)</td>
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</table>

<table>
<thead>
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<th>Refinement</th>
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<tr>
<td>No. reflections</td>
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<tr>
<td>R-work/R-free</td>
<td>11.5/13.8</td>
</tr>
<tr>
<td>No. of water</td>
<td>412</td>
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<tr>
<td>r.m.s. deviations</td>
<td></td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
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</tr>
<tr>
<td>Bond angles (°)</td>
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</tr>
</tbody>
</table>
Table S3, related to Figure 3: Dihedral angles $\chi_1$ and $\chi_2$ of residue 105 in apoTEM-1, TEM-1 acyl-enzyme intermediate, PSE-4 and cTEM-17m.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$\chi_1$</th>
<th>$\chi_2$</th>
<th>Rotamer $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoTEM-1 (1ZG4)</td>
<td>191°</td>
<td>69°</td>
<td>$t80^\circ$</td>
</tr>
<tr>
<td>apoTEM-1 (1BTL)</td>
<td>194°</td>
<td>70°</td>
<td>$t80^\circ$</td>
</tr>
<tr>
<td>acyl-TEM-1 (1FQG)</td>
<td>192°</td>
<td>71°</td>
<td>$t80^\circ$</td>
</tr>
<tr>
<td>apoPSE-4 (1G68)</td>
<td>191°</td>
<td>76°</td>
<td>$t80^\circ$</td>
</tr>
<tr>
<td>apo cTEM-17m (4ID4)</td>
<td>305°</td>
<td>124°</td>
<td>$m-30^\circ$</td>
</tr>
</tbody>
</table>

$^a$ Nomenclature according to the ‘Penultimate rotamer library’ (Lovell, et al., 2000). The $t80^\circ$ conformation has been assigned in all native and mutant apoenzymes of TEM-1 and PSE-4 and in their ligand-bound structures, with the exception of structures with bound boronate inhibitor or $\beta$-lactamase inhibitor protein (BLIP) where the $m-30^\circ$ conformer has been assigned.
Figure S1, related to figure 2: $^{15}$N-CPMG relaxation dispersion curves for TEM-1 (A) and PSE-4 (B) at 800MHz (full line) and 500MHz (dashed line). The residue number appears above each plot.
Figure S2, related to figure 2: Residue-specific transverse relaxation rates ($R_2$ for $\tau_{cp} = 0.625$ ms) for TEM-1 (A), PSE-4 (B), and cTEM-17m (C). $R_2$ values were determined using a 20-ms constant relaxation time version of the TROSY $^{15}$N-CPMG experiment where the delay between CPMG 180° refocusing pulses was set to 0.625 ms. Experiments were performed at 18.8 T and 298 K, pH 6.8. Residues with $R_2$ values > 1 standard deviation above the mean $R_2$ value (dashed lines) are labeled. Relaxation rates above the overall mean value have been proposed to indicate μs-ms motions that are not suppressed even at the fastest CPMG pulsing rates used in the relaxation dispersion experiment (Doucet, et al., 2011). While the specific residues with elevated relaxation rates generally differed, 10 possessed elevated relaxation rates in both TEM-1 and PSE-4 but not cTEM-17m, including Thr71, Val216 and Gly238. Residue 71 is next to the catalytic Ser70, Val216 influences substrate binding and Gly238 modulates the size of the active-site cavity (Huletsky, et al., 1993). In cTEM-17m, Met69, which precedes Ser70, shows an elevated relaxation rate. As Val216 and Gly238 were unassigned in cTEM-17m, those $R_2$ values cannot be compared.
Figure S3, related to figure 3: Surface charges of the 150-190 region in TEM-1 (top), or cTEM-17 and PSE-4. The interior surface forms one of the active-site walls. Surface charge was generated using the APBS tool in PyMOL and colored according to a red (negatively charged) to blue (positively charged) scale. The charge difference was most significant on the exterior face, which is more positively charged for PSE-4 and cTEM-17m than TEM-1, while the interior face – forming one of the active-site walls – remained generally negatively charged as in TEM-1.
Figure S4, related to figure 2: $^1$H-$^{15}$N HSQC spectra for TEM-1 (Savard and Gagné, 2006), cTEM-17m (Morin, et al., 2010) and an overlay of TEM-1/cTEM-17m.
Figure S5, related to figure 3: Crystallographic B-factors for cTEM-17m. The deviation from the overall B-factor is colored in a blue to red scheme, where blue is smaller than the mean - 2SD and red is greater than the mean + 2SD of the overall B-factor.
Figure S6, related to figure 2: $^{15}$N-CPMG relaxation dispersion curves for cTEM-17m at 800MHz (full line) and 600MHz (dashed line). The residue number appears above each plot.
SUPPLEMENTAL REFERENCES


