\(\beta\)-Lactamases Evolve against Antibiotics by Acquiring Large Active-Site Electric Fields

Zhe Ji* and Steven G. Boxer*

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ABSTRACT: A compound bound covalently to an enzyme active site can act either as a substrate if the covalent linkage is readily broken up by the enzyme or as an inhibitor if the bond dissociates slowly. We tracked the reactivity of such bonds associated with the rise of the resistance to penicillin G (PenG) in protein evolution from penicillin-binding proteins (PBPs) to TEM \(\beta\)-lactamases and with the development of avibactam (Avb) to overcome the resistance. We found that the ester linkage in PBP–PenG is resistant to hydrolysis mainly due to the small electric fields present in the protein active site. Conversely, the same linkage in the descendant TEM–PenG experiences large electric fields that stabilize the more charge-separated transition state and thus lower the free energy barrier to hydrolysis. Specifically, the electric fields were improved from \(-9\) to \(-140\) MV/cm in an ancient evolution dating back billions of years, contributing 5 orders of magnitude rate acceleration. This trend continues today in the nullification of newly developed antibiotic drugs. The fast linkage hydrolysis acquired from evolution is counteracted by the upgrade of PenG to Avb whose linkage escapes from the hydrolysis by returning to a low-field environment. Using the framework of electrostatic catalysis, the electric field, an observable from vibrational spectroscopy, provides a unifying physical metric to understand protein evolution and to guide the design of covalent drugs.

INTRODUCTION

Resistance to \(\beta\)-lactam antibiotics arises from the remarkable proficiency of \(\beta\)-lactamases in breaking up \(\beta\)-lactam rings.\(^1\)\(^-\)\(^3\) TEM-1, a prototypical \(\beta\)-lactamase, has perfected the degradation of penicillin G (PenG) by first using the hydroxyl group of S70 to open the \(\beta\)-lactam ring, forming an ester bond that ties PenG in the covalent complex TEM–PenG (Figure 1A).\(^1\) The ester bond is then subject to a nucelophic attack from a well-positioned water molecule assisted by a general base E166. The rapid hydrolysis of the ester linkage quickly releases TEM-1 from the covalent adduct so that another catalytic transformation can queue up. By contrast, the same ester hydrolysis is sluggish in the active site of penicillin-binding proteins (PBPs), trapping PBPs in the covalent complex PBP–PenG (Figure 1B).\(^5\)\(^-\)\(^7\) The covalently trapped PBPs can no longer act as DD-peptidases in the biosynthesis of the bacterial peptidoglycan cell wall, leading to the antibiotic effect of PenG. Thus, PenG switches its role from being a covalent inhibitor for PBP to being a substrate for TEM-1 due to the disparity in the rate of ester hydrolysis.

This shift in hydrolysis rate is an outcome of protein evolution, with a history far beyond the antibiotic era, dating back billions of years when ancient microbes acquired the capability to secrete PenG to kill other microbes for survival.\(^8\) In response, defense against PenG emerged from the evolution of PBPs into \(\beta\)-lactamases, which confers the enzyme machinery with a hydrolysis apparatus.\(^6\) Compared with PBP-A, currently the closest known evolutionary ancestor,\(^9\) TEM-1 retains the protein scaffold, consensus sequences in the active site, and fast bonding to PenG yet features a longer \(\Omega\)-loop bearing the key E166 in the place of L158 for carrying out the ester hydrolysis.\(^10\) To circumvent this biochemical defense harnessed by pathogens, avibactam (Avb) has been developed as a drug that effectively reverses protein evolution and traps TEM-1 in the covalent complex TEM–Avb (Figure 1B).\(^11\)\(^,\)\(^12\) The upgrade from PenG to Avb prohibits TEM-1 from executing the hydrolysis necessary to free TEM from covalent inhibition. From the hydrolytically inert PBP–PenG to the labile TEM–PenG, and back to the inert TEM–Avb, this reverse direction in modulating the hydrolysis rate manifests the competition between protein evolution and drug development (Figure 1B).

What makes the hydrolysis slow for PBP–PenG and TEM–Avb compared with TEM–PenG? A previous study has installed the same hydrolytic apparatus TEM-1 uses into PBP-A by making the L158E mutation (Figure 1B).\(^9\) The incorporated glutamate and the essential water molecule are almost perfectly superimposed with those of TEM-1 (Figure S1);\(^10\) however, the ester hydrolysis of PBP–PenG is only enhanced by 90-fold, still 5 orders of magnitude slower than that of TEM–PenG (Table 1).\(^9\) The similar inertness of TEM–Avb was ascribed to the
We find that this is insufficient to explain a 10 orders of magnitude gap in their hydrolysis rates (Table 1 and Text S1 in the Supporting Information). In this work, we examined the hydrolysis reaction within the framework of enzyme electrostatic catalysis. Different from applying external electric fields, enzymes organize charges and dipoles into a distinct pattern that imposes stabilizing electric fields onto the charges developed in the transition state. We note that the fields present in matter are typically much larger than those that can be achieved by applying an external electric field, unless that field is on the molecular length scale. The preferential stabilization of the transition state against the reactant state lowers the free energy barrier and accelerates the reaction. The rate limiting step of the linkage hydrolysis discussed here is the conversion of the C\(^\equiv\)O to an oxyanion intermediate C\(^−\)O\(^−\) (Figure 1A), with a transition state more charge-separated along the bond axis (Figure 1C). The corresponding increase in dipole moment or reaction difference dipole, \(\Delta\mu_{\text{rxn}}\), interacts with the electric fields \(\vec{F}\) experienced by the C\(\equiv\)O to produce a lowering of the free energy barrier as \(\Delta\Delta G^\ddagger = -\vec{F} \cdot \Delta\mu_{\text{rxn}}\). For TEM and many other enzymes, the electric fields \(\vec{F}\) are mainly exerted by two conservative backbone amides acting as H-bond donors (Figure 1A). By mutating one of the backbone amides to an ester, we knocked out one of these key H-bonds and thus perturbed the electrostatic interactions imposed onto the reactive C\(\equiv\)O. The corresponding decrease in electric fields was measured using the vibrational Stark effect.

Table 1. Kinetic Parameters for Linkage Hydrolysis

<table>
<thead>
<tr>
<th>Linkage Hydrolysis Rate (s(^{-1}))</th>
<th>(\Delta G^\ddagger) (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PenG(^\ddagger)</td>
<td>TEM-1 2769 [2697, 2843] 12.66 [12.65, 12.68]</td>
</tr>
<tr>
<td>TEM-1 A237Y(^e)</td>
<td>4.80 [4.75, 4.87] 16.40 [16.39, 16.41]</td>
</tr>
<tr>
<td>TEM-1 A237E</td>
<td>1466 [1442, 1500] 13.03 [13.02, 13.06]</td>
</tr>
<tr>
<td>TEM-1 A237R</td>
<td>5462 [5191, 5773] 12.26 [12.23, 12.29]</td>
</tr>
<tr>
<td>TEM-1 A237W</td>
<td>436 [432, 441] 13.75 [13.74, 13.75]</td>
</tr>
<tr>
<td>PBP-A</td>
<td>1.6 \times 10^{-4} 22.46</td>
</tr>
<tr>
<td>PBP-A L158E</td>
<td>1.4 \times 10^{-2} 19.83</td>
</tr>
<tr>
<td>Avb</td>
<td>TEM-1 (8 ± 1) \times 10^{-8} 26.94 ± 0.07</td>
</tr>
<tr>
<td>Ctx(^d)</td>
<td>TEM-1 93.1 [38.7, 248.9] 14.76 [14.18, 15.28]</td>
</tr>
</tbody>
</table>

*References 9 and 13. \(^e\)Reference 14. Ctx stands for cefotaxime.*

Figure 1. Hydrolysis of carbonyl linkages in protein covalent adducts. (A) Mechanism of TEM−PenG hydrolysis. The ester linkage is attacked by a water deprotonated by E166 forming an oxyanion intermediate, which quickly releases penicilloic acid as the product. (B) The rate of hydrolysis determines whether the attached molecule is a covalent inhibitor or a substrate. PBP-A hydrolyzes the ester linkage slowly with a nonpolar L158 at the site of the basic E166 in TEM-1. The hydrolysis is not much improved with a L158E mutation. The protein evolution from PBP-A to TEM-1 improved the linkage hydrolysis, turning PenG from an inhibitor to a substrate. The development of Avb is the reverse process, where the impaired linkage hydrolysis makes Avb a covalent drug. (C) Model of electrostatic catalysis. The electric fields produced by two H-bonds stabilize the transition state more than the reactant because the transition state experiences more charge separation along the C\(\equiv\)O bond.
as observed from infrared (IR) spectroscopy. By quantifying the electrostatic contribution to the shift in hydrolysis rates, we find that the concept of electrostatic catalysis provides a unifying framework governing the competition between protein evolution and covalent drug development, spanning the past, present, and perhaps future.

**RESULTS AND DISCUSSION**

We chose the H-bond donated by the backbone amide of A237 as the focus of our studies (Figure 2A) because its side chain is solvent-exposed and can be replaced with tyrosine with little functional or structural consequence. After obtaining the A237Y mutant of TEM-1, we expressed its ester counterpart (A237Y') with a backbone ester replacing the backbone amide using amber suppression as described in earlier work (Figure 2A). The backbone ester in A237Y' is found to be unusually susceptible to hydrolysis even at a neutral pH because the C=O of the backbone ester itself sits in an oxyanion hole (Figure S2), another manifestation of electrostatic catalysis. Coupled with the oxyanion hole is a potentially well-positioned water acting as the nucleophile (Figure S2). The water can be deprotonated by E166, the same general base used for linkage hydrolysis, mediated by a water chain in the active site for proton relay (Figure S2). This mechanism is supported by a significantly slower hydrolysis of the backbone ester when the base E166 is removed in the E166N mutant or when the water chain is disrupted by a covalently bound Avb (Figures S2–S4).

With the A237Y mutants obtained, we measured the linkage hydrolysis of TEM−PenG by performing full-time kinetic studies and steady-state MS (Table 1).11 A237Y' was found to react 230-fold slower than A237Y, while A237Y is only 2.5-fold slower than the wildtype (WT). This observation indicates that the H-bond removed by the ester backbone (Figure 2A) imposes a key electrostatic interaction facilitating the hydrolysis of the ester linkage.

To probe electric fields experienced by the C=O in the linkage, we measured the IR spectra of the C=O. According to the linear vibrational Stark effect, the vibrational frequency shifts linearly with the magnitude of electric fields projected on the bond.6,29 We calibrated this linear correlation using ethyl acetate as a model compound15,17 of the ester linkage in PBP−PenG and TEM−PenG. To model the carbamate linkage in TEM−Avb, methyl piperidine-1-carboxylate was employed as the model compound.15 Once the linkage C=O probes are calibrated, their measured vibrational frequency can be translated into the magnitude of electric fields in protein active sites.

To carry out spectroscopic studies of the proteins, we made TEM−PenG using a E166N mutant that is deprived of the general base necessary for the hydrolysis of the ester linkage, thus trapping PenG in the covalent complex,14 as evidenced by mass spectrometry (Table S1). A separate sample containing a 13C=O in the ester linkage was prepared using isotopically labeled PenG.14 By subtracting the IR absorption spectrum of the 13C TEM−PenG from that of the 12C TEM−PenG, we obtained a difference spectrum where the protein background cancels out,14 allowing us to distinguish the positive peaks of the 13C=C=O vibration that are ∼45 cm⁻¹ apart from the negative peaks of the 12C=C=O vibration (Figure S5). By fitting positive and negative Lorentzian−Gaussian peaks to the difference spectra (Table S2),15 the 12C=O vibrational peaks were obtained as the positive components, and these are shown in Figure 2B.

The WT exhibits three peaks with frequencies of 1716, 1688, and 1654 cm⁻¹, which were mapped to electric fields of −49, −90, and −140 MV/cm, respectively, using the calibration curve (the negative sign stands for stabilization of the C=O dipole) (Figure 2B). The observed peak multiplicity indicates a heterogeneous electrostatic environment surrounding the C=O. Given only one conformation of the ester linkage in the crystal structure of TEM−PenG,30 the electrostatic heterogeneity may originate from variations in the H-bond length far below the precision of the coordinates obtained from crystallography or configurations inaccessible to low-temperature crystals. Vibrational spectroscopy not only provides a sensitive readout of electric fields but also probes the local electrostatics in more native conditions.

Compared to the WT TEM−PenG, the A237Y mutant was observed to maintain the three vibration peaks with slight shifts...
electric field is absent for A237Y frequency peak corresponding to the largest (most negative) backbone amide of A237. Markedly, the reddest (lowest (Figure 2B), as do other A237 canonical mutants (Figure S6), indicating minimal perturbations on the C\textsuperscript{O}'s electrostatic repulsion against the largest electric field experienced by the linkage C\textsuperscript{O} (Text S2 in the Supporting Information). The greater protein environment may also have contributed exponentially more than a smaller field to field improvement (Table S3). Along the evolutionary trajectory from PBP-A to TEM-1 in acquiring linkage hydrolysis, the electric field contributes dramatically increases from \(-10\) to \(-140\) MV/cm (Figure 2D). Although the shortened H-bonds can contribute substantially to field improvement (Text S2 in the Supporting Information), the greater protein environment may also have been optimized by evolution to produce larger electric fields (Text S3 in the Supporting Information).

The upgrade from PenG to Avb effectively reverses protein evolution by diminishing the electric fields. The linkage C\textsuperscript{O}O in TEM-Avb was found to experience an ensemble of fields ranging from \(-40\) to \(-97\) MV/cm (Figure 2D).\textsuperscript{13} The highest field reduces from \(-140\) MV/cm for TEM-PenG to \(-97\) MV/cm for TEM-Avb, offsetting the evolution from PBP-A to TEM-1 in acquiring high fields. This reversed trend is also reflected in the structural overlay of TEM-PenG\textsuperscript{13} and TEM-Avb\textsuperscript{13} (Figure 2C). The linkage C\textsuperscript{O}O in TEM-Avb is found to deviate from the best orientation for accepting the H-bond from the A237 backbone amide, almost returning to the original position of the linkage C\textsuperscript{O}O in PBP-PenG. As a result, the H-bond length elongates from 2.8(1) to 3.0(1) Å (Figure 2C).

Based on the rates of linkage hydrolysis obtained from kinetic studies, we calculated the free energy barriers (\(\Delta G^\ddagger\)) (Table 1) and plotted them against the magnitudes of the largest electric fields (F) for the WT, A237 canonical mutants, and the ester mutant A237Y\textsuperscript{e} of TEM-PenG (Figure 3A). According to the electrostatic catalysis model, a linear correlation was fitted as \(\Delta G^\ddagger = 1.7 F + 24.3\) kcal/mol (the green solid line in Figure 3A), with \(\Delta G^\ddagger = 1.4 + 24.3\) kcal/mol without stabilizing electric fields in the active site. The slope gives \(\Delta \mu_{\text{intr}} = 1.7\) D, a measure of the catalyzability of the reaction by electric fields.\textsuperscript{16} We define this line “the line of electrostatic catalysis” for this reaction, from...
which the $-140 \text{ MV/cm} \left(\sim -6.7 \text{ kcal/mol/D}\right)$ electric field in WT TEM–PenG contributes a rate acceleration by 8 orders of magnitude ($\Delta \Delta G^\ddagger = -11 \text{ kcal/mol}$).

For PBP–PenG, the L158E mutation exhibits $\Delta \Delta G^\ddagger = -2.6 \text{ kcal/mol}$ compared with the nucleophile-deficient WT (Figure 3A, @), according to their linkage hydrolysis rates available in literature (Table 1). With the values of $\Delta G^\ddagger$ and $P$ for PBP–PenG obtained (Text S4 in the Supporting Information), the L158E mutant (solid green hexagon) is found to align strikingly well to the line of electrostatic catalysis obtained for TEM–PenG (the green solid line in Figure 3A). This result implies that the general base and the nucleophilic water installed by the L158E mutation in PBP-A indeed function as well as those in TEM-1; what prevents PBP-A from performing hydrolysis at the level of TEM-1 is the big gap in their electric fields. From PBP-A-L158E to TEM-1, the increase in field magnitude by 81 MV/cm contributes to $\Delta \Delta G^\ddagger = -6.6 \text{ kcal/mol}$ (Figure 3A, @), meaning that the active site electric field accounts for 67% of the total $\Delta \Delta G^\ddagger$ due to evolution (Figure 3A, @ + @).

The rate of linkage hydrolysis for WT TEM–Avb was measured to be $8 \times 10^{-8} \text{ s}^{-1}$ (Figure S7), which can be translated to $\Delta G^\ddagger = 14.3 \text{ kcal/mol}$ with respect to WT TEM–PenG (Figure 3A, @ + @). Extrapolating TEM–Avb to zero field (Text S5 in the Supporting Information) gives $\Delta G^\ddagger_{0-0} = 33.5 \text{ kcal/mol}$ (Figure 3A), which is higher than that for TEM–PenG by $\Delta \Delta G^\ddagger_{0-0} = 9.2 \text{ kcal/mol}$. If we draw a line passing through TEM–Avb’s zero-field point but with the slope of TEM–PenG’s line of electrostatic catalysis (the green dashed line in Figure 3A), then we obtain a hypothetical covalent adduct that features TEM–Avb’s intrinsic (non-electrostatic) reactivity but TEM–PenG’s sensitivity to electrostatic catalysis. @ in Figure 3A connecting the two green lines represents the gap in intrinsic reactivity; thus, its $\Delta \Delta G^\ddagger = \Delta \Delta G^\ddagger_{0-0} = 9.2 \text{ kcal/mol}$. This can be ascribed to the lower electrophilicity of the carbamate linkage in TEM–Avb compared with that of the ester linkage in TEM–PenG (Text S1 in the Supporting Information) as well as the lower nucleophilicity of the water due to a protonated E166 in TEM–Avb (Figures S8–S10 and Text S6 in the Supporting Information). The rest, 5.1 kcal/mol TEM–Avb’s higher stability, can be ascribed to the electrostatic effect (Figure 3A, @).

In Figure 3B, we add the results of our previous studies on the modern (clinical) evolution from TEM-1 to TEM-52 driven by the shift in substrate scope from PenG to cefotaxime (Ctx). The ester C=O in the linkage of TEM–Ctx experiences an improved electric field from $-123 \text{ to } -141 \text{ MV/cm}$, leading to a $\Delta G^\ddagger$ decrease by 1.0 kcal/mol. The trend for TEM–Ctx (Figure 3B, @) closely follows the line of electrostatic catalysis described for TEM–PenG (Figure 3B, @), indicating that the physical basis for evolution on a planetary time scale can be extrapolated to the evolution in the modern era. The ancient evolution from PBP-A to TEM-1 and the modern evolution from TEM-1 to TEM-52 can be described by a common physical metric—the electric field at the active site. The billion-year-old ancient evolution from PBP-A to TEM-1 acquired $10^{1.5}$-fold rate acceleration by improving the electric fields by $-61 \text{ MV/cm}$; the modern evolution from TEM-1 to TEM-52 in the last decades continued this trend by acquiring $10^{0.7}$-fold rate acceleration for the new substrate Ctx through a $-18 \text{ MV/cm}$ improvement in electric fields. Whatever the time scale, protein evolution selects for mutations leading to larger electric fields for increased catalytic function.

Covalent drugs are designed to exhibit sluggish linkage breakup. The upgrade from a substrate to a covalent inhibitor that bonds to the same enzyme active site requires a reversal of the effects of protein evolution. Lesser nucleophilicity of nucleophiles and lesser electrophilicity of linkages can vertically shift up the line of electrostatic catalysis (Figure 3B, @). This effect can be compounded by positioning the reactive group in a low-field environment to escape from electrostatic catalysis (Figure 3B, @). The physical insights provided by this work may be applicable to predict the future pattern of the interconversion between substrates and inhibitors. New enzymes that rapidly hydrolize TEM–Avb may emerge from enhanced nucleophilicity of water and electric fields, continuing the cycle of a competition between $\beta$-lactamase evolution and antibiotic development (Figure 3B, @ and @).

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.2c10791.

Experimental details including protein expression and purification, mass spectrometry, vibrational spectroscopy, and kinetics of TEM–Avb linkage hydrolysis, and MD simulation methods (PDF).

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**Notes**

The authors declare no competing financial interest.

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**ABBREVIATIONS**

PenG penicillin G

Avb avibactam

PBP penicillin-binding proteins

Ctx cefotaxime

WT wildtype

IR infrared

**REFERENCES**


Supporting Information

β-Lactamases evolve against antibiotics by acquiring large active-site electric fields

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**Materials and Methods**

**Plasmid Construction**

The pBAD plasmids containing the gene for TEM-1 β-lactamase and PBP-A were generously provided by the Patrice Soumillion lab at the Université Catholique de Louvain. The details of the plasmid for TEM-1 β-lactamase were described in our previous work\(^1\). The PBP-A plasmid carries a tetracycline resistance gene. A DsbA signal sequence was added to the N-terminus, followed by a His tag\(^2\). A ssrA degradation tag was added to the C-terminus. Point mutations were made using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent) according to the manufacturer’s protocols.

Site-specific incorporation of a backbone ester was achieved using an aminoacyl-tRNA synthetase (aaRS)\(^3\). A pUltra plasmid containing the aaRS gene was generously provided by the Peter G. Schultz lab at the Scripps Research Institute.

**DNA Sequence**

The sequence of TEM-1 can be found in our previous work\(^1\).

**PBP-A**

*Codon for L158 in bold*

```
ATGAAAAGATTTTTGCTGCGCTGCTGTGGTTTAGGTGTTAGGCCTGCTGACCACATCGGCCGCCCATGG
ACCATCATCATCATCAACCCGACGACGACGCTGCGCAGCCGAAACGCCCCCTGACGATCTGCAACA
ACAAATCCAAACACCTGCTGCGACCGCGACGTGACGCTGCTGAGCTGCTGCTGCTGCTGCTGCTG
GATTAGCGGCGCCTCTCTCTGAAATGTCGTGCGGTGACAAGGTTTCTCGGCCGCGGCTTCACTTAAAT
TCGGATCTTCTGCGCTGGTTTTTTCAAGCGGTTGATGAAGTGGTCGTCACGCTGCAGGAACGAGCC
GACCATGCGCCCGGACCCTGATTGCCGCCGAAAAGCAGGCACCCCTGCACTATCAAAAAACGAAACTC
CAATACGACGCTCCTGCGGACCGCAGCCGAACTGAACTGAACGAAATTTGAGAGGTTGCTAGA
AAATACCGTCTCAACAAATCGCGCTGCGGCGGCTTAA
```

**Protein Sequence**

The sequence of TEM-1 can be found in our previous work\(^1\).
PBP-A

L158 in bold; DsbA signal peptide in purple; ssrA degradation tag in green

MKKIWLALAGLVLALFSAAMDHHHHHTPTSTLPERRPLTNLQQQLQQLVSRQPNLTAAGLYFFNL
DSGASLNVGDDQVFPAASTIKFPLVAFFKAVDEGRVTCLRERTMPLDIAPEAGTLQYKQKNS
QYAALEVAELMIISDTNATNMIDRLGAAELNQQFQEWGENTEINNLPDMKGTNTTPRDL
LATLMKIGQGEILSPRSRDLDLIMRRTVNTLPPGLGKATIAHKGDIGIVVGDAVMVM
PNGQRYVAAMVVRPYNDPRGSELIRQVSMVFQAEEKLPPTGANDENYALAAV

Protein Expression and Purification

The protocols for expressing and purifying TEM-1 proteins, including the A237Yc mutant using amber suppression can be found in our previous work1.

For the expression of PBP-A, we followed the protocol in literature2. Specifically, the pBAD plasmid was transformed into chemically competent TOP10 Escherichia coli cells (Invitrogen) using selection with 12.5 μg/mL tetracycline (Sigma) on Luria Broth (Fisher) agar plates. A single colony of the transformed cells was grown into 1 mL culture for 8 h and further into 50 mL cultures overnight using Luria Broth with 12.5 μg/mL tetracycline at 37°C. 50 mL of an overnight culture was used to inoculate 1 L of Luria Broth with 12.5 μg/mL tetracycline and 1 g/L glucose (Sigma), shaking at 140 rpm and 37°C until they reached an OD600 ~ 0.6. The media was centrifuged at 6,000 × g for 20 mins and the cell pellet was redispersed in fresh Luria Broth media with 12.5 μg/mL tetracycline. Protein expression was induced with 1 g/L L-(+)-arabinose (Sigma) and grown for 5 h at 30°C. Cells were harvested by centrifugation at 6,000 × g for 20 mins and resuspended in a lysis buffer [50 mM potassium phosphate (KPi), 20 mM imidazole, 500 mM NaCl, 10% (v/v) glycerol, pH 7.4]. Cells were lysed with a homogenizer (Avestin EmulsiFlex-C3), and the lysate was a centrifuged twice for 90 min each at 15,000 × g. The crude protein in the supernatant was filtered through an 0.45 μm filter membrane before being loaded onto a Ni-NTA affinity resin (QIAGEN) column equilibrated with the lysis buffer. The column was washed with 10 column volumes of the lysis buffer before protein elution with the washing buffer (50 mM KPi, 50 mM imidazole, 500 mM NaCl, pH 7.4) and the elution buffer (50 mM KPi, 200 mM imidazole, 500 mM NaCl, pH 7.4). Further purification was performed using anion exchange chromatography on a 5 mL HiTrap-Q HP (GE Healthcare) column and eluted using a 0-25% gradient of buffer A (25 mM Tris-HCl, 25 mM NaCl, pH 8.4) and buffer B (25 mM Tris-HCl, 1 M NaCl, pH 8.4) over 25 column volumes. Purified protein was exchanged into a cryoprotecting storage buffer (50 mM KPi, 100 mM NaCl, 10% (v/v) glycerol, pH 7.4) for long-term storage at -80°C. A typical yield is 2.5 mg of protein from 1 L of media. The identity and purity of the proteins were confirmed with HPLC-MS. The expected and observed masses are summarized in Table S2.
Mass Spectrometry

Mass spectrometry was performed in the Stanford University Mass Spectrometry facility on a Waters Single Quadrupole Mass Spectrometer and Acquity H Class UPLC equipped with a reverse-phase C8 column (Zorbax 300SB-C8, Rapid Resolution 2.1 × 50 mm 3.5u with 300SB-C8 Narrow=Bore Guard column 2.1 × 12.5mm 5u). The LC mobile phases consist of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. ESI was performed under standard cone voltage. The mass was recorded in ESI+ mode in the range of 50-2000 m/z. Data deconvolution and analysis were performed using the Intact Mass software (Protein Metrics).

Isotope-Edited FTIR Spectroscopy

The proteins (TEM-1-E166N or PBP-A) were concentrated to a volume of 60 µL at a concentration of 2.3 mM using a 10,000 Da MWCO Amicon spin filter (Millipore). The proteins were then solvent exchanged with a D2O buffer containing 100 mM NaCl and 50 mM KPi (pD 7.4) using a Micro Bio-Spin P-6 gel column (Bio-Rad). The protein solution was allowed to sit at 4°C overnight for completing H-D exchange. Afterwards, the protein solution was solvent exchanged again with the D2O buffer before splitting into two portions of equal volume (~ 30 µL). PenG, both 13C-labeled and unlabeled, were prepared in the same D2O buffer at a concentration of 25 mM, respectively. 4 µL of the 13C-labeled and unlabeled PenG solution were added into each portion of the protein solution, respectively. The two samples were mixed using a pipette and spun down. The protein samples were directly used for FTIR measurements, except for A237Y used for preparing the covalent complexes with PenG, which was incubated at room temperature for 3 h due to the slow formation of the covalent linkage. Mass spectra were taken for samples of covalent complexes (Table S2). These samples were quenched by using MeOH/H2O (1:1, v/v) both before and after the FTIR measurements, which confirmed the formation and stability of the covalent bonds (Table S2).

Infrared spectra were recorded using a Bruker Vertex 70 equipped with a liquid nitrogen-cooled mercury cadmium telluride detector and under constant purging of the sample chamber with dry air, similarly to previous works. 20 µL of the samples were loaded into a demountable cell composed of two CaF2 optical windows (19.05 mm diameter, 3 mm thickness, Lambda Research Optics). The windows were separated by two Teflon spacers of 25 and 50 µm thickness. Before FTIR measurement, the sample chamber was purged for 10 min to remove gaseous CO2, and then 512 scans were acquired and averaged to obtain each transmission interferogram. Spectra were recorded in a window of 4000–1000 cm−1 with 1 cm−1 resolution. Nine spectra were collected for each sample (three for each cell orientation). The IR spectra of free Pen-G, both 13C-labeled and unlabeled, were also collected for use as references.

The procedure for data processing has been described in detail in previous works. Briefly, the raw transmission spectra were converted to absorption spectra. To remove protein background, difference FTIR spectra were generated by subtracting the protein spectra of the 13C-labeled PenG
from that of the unlabeled one. A scaling factor close to unity was typically used to compensate for slight differences in sample concentration and pathlength. The resulting spectra were further processed by removing the broad peaks associated with the unbound, free PenG, and the weak peaks associated with H-D exchange. The latter were obtained by taking the difference from the 9 spectra of the same sample (either the $^{13}$C-labeled or the unlabeled). Finally, the spectra were baselined guided by the offset (35-55 cm$^{-1}$) between the positive and negative peaks, the frequency shift due to $^{13}$C-labeling. This process was repeated for the 9 spectra taken for each sample to ensure consistency. To deconvolve the difference spectra where positive $^{12}$C peaks overlap with the negative $^{13}$C peaks, the difference spectra were fitted into component peaks as Gaussian-Lorentzian sums$^6$, using a script presented in our previous work$^1$.

**Kinetics Measurements on the Carbamate Linkage Hydrolysis for TEM–Avb**

**General.** We developed an assay to monitor the hydrolysis of the carbamate linkage in TEM–Avb. We first prepared a mixture of TEM-1 and Avb sodium (Advanced ChemBlocks), where Avb was in a slight molar excess (e.g., 1.4 eq). After the equilibrium between TEM•Avb and TEM–Avb was established, there was still free Avb (0.4 eq) in solution. The reaction scheme (c.f. Figure 1A) is:

\[
\begin{align*}
\text{TEM} + \text{Avb} \rightleftharpoons \text{TEM}•\text{Avb} & \quad k_{cbm} \quad \text{TEM–Avb} \quad k_{hyd(slow)} \quad \text{TEM} + \text{Product} \\
\end{align*}
\]

\[\text{eq. 1.}\]

During the incubation, the hydrolysis of the carbamate linkage frees TEM-1 from the covalent adduct for forming new TEM–Avb, which causes a drop in the concentration of the free Avb. The free Avb was collected by spin filtration and its concentration was quantified by a separate inhibition assay as detailed below. The low ratio of free Avb (0.4 eq) to TEM makes the very slow process more observable. Multiple collections and measurements of free Avb over the course of incubation allows for determining the rate of carbamate linkage hydrolysis.

**Hydrolysis experiment.** TEM-1 was prepared in 50 mM KPi (pH 7.0) at a concentration of 20 µM, into which Avb was added to reach a final concentration of 28 µM. The total volume of the mixture was 1.2 mL. The mixture was incubated at room temperature. From time to time, 150 µL aliquots were taken and filtrated using a 3,000 Da MWCO Amicon spin filter (Millipore). Typically, ~100 µL filtrate can be collected, which was free of TEM-1 or TEM–Avb and only contains free Avb. The complete removal of TEM and TEM–Avb was evidenced by a dilution experiment$^7$, which showed no recovery of the enzyme activity.

**Inhibition assay.** The filtrate was measured by an inhibition experiment as described in our previous work$^1$. To measure Avb inhibition kinetics, nitrocefin, a TEM substrate, was mixed with Avb and then together mixed with $\beta$-lactamases. After the equilibrium between TEM•Avb and TEM–Avb was established, the rate of nitrocefin turnover becomes constant over time and follows:
\[ v_s = \frac{k_{\text{cat}}[E]_0[S]}{K_M + \frac{[I]}{K_i^*} + \frac{[S]}{K_M}} \]  

where \( v_s \) is the rate of nitrocefin turnover. \( E_0 \) is the concentration of TEM-1 added to the mixture, \( S \) for the substrate, nitrocefin, \( I \) for the inhibitor, Avb. \( K_i^* \) is the overall dissociation constant for Avb. \( k_{\text{cat}} \) and \( K_M \) are the Michaelis-Menten parameters for the degradation of nitrocefin by TEM-1. \( K_i^* \), \( k_{\text{cat}} \), and \( K_M \) were taken from our previous work \(^1\). Nitrocefin is in large excess, so \([S]\) can be treated as a constant. Therefore, \( v_s \) becomes a function only of \([I]\).

The WT was prepared in 50 mM KPi (pH 7.0) at a final concentration of 0.5 nM, into which the filtrate was added with 8-fold dilution ([I] = 0.5-1.0 \( \mu \)M depending on the hydrolysis progress). Nitrocefin was added to the mixture at a final concentration of 200 \( \mu \)M. The mixture was monitored at 486 nm on a Lambda 365 (Perkin-Elmer) UV-Vis spectrometer until enough data in the linear region had been collected. The final slope \( v_s \) was analyzed for calculating \([I]\) using eq. 2. The carbamate linkage hydrolysis is a zeroth-order process due to the constant concentration of TEM–Avb. Therefore, the free Avb concentration follows a linear correlation with incubation time, and the slope divided by the concentration of TEM–Avb provides the first-order rate constant of the carbamate hydrolysis (Figure S7).

Polarizable MD Simulations

Polarizable molecular dynamics simulations were performed using Tinker\(^9\) (ref. 8) with the AMOEBA\(^{18}\) force field.\(^9,10\) The serine-AVB covalent adduct was parameterized with acetyl and dimethyl amine caps using Poltype\(^\dagger\),\(^11\) in which electrostatic parameters were obtained from fitting the electrostatic potential of 6 conformers at the MP2/aug-cc-pvDz level, and torsion parameters were refined based on dihedral scans at the WB97x-d/6-311+G** level of theory with steps of 20–30° (depending on the number of parameters to be fit). The parameters the entire capped serine backbone were taken from the AMOEBA\(^{18}\) force field (kept fixed during the parameterization) to ensure compatibility with the force field, while the parameters for the sidechain were refined. To ensure that the residue carried a total charge of -1, the charge of Cα atom was adjusted accordingly (by ca. 0.05). The crystal structure of the covalent adduct of TEM1 and AVB (8DE0)\(^1\) was taken as a starting point, minimized, and equilibrated as NVT (Bussi thermostat; Ewald and vdw cutoffs of 7 and 12 Å; induced dipole threshold of 10\(^-4\) D; 1 fs steps) and NPT (Monte Carlo barostat; otherwise, similar conditions) ensembles for 500 ps each. Three production runs were performed over 10 ns for each system. Electric fields along the AVB C=O bond were determined as the averaged fields on the C and O atoms projected along the C=O bond axis, as reported previously.\(^12\)
Table S1. Masses and extinction coefficients of TEM-1 and PBP-A

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bProteins of ~ 30 kDa have a systematic error within +15 Da, depending on the instrument status.

cHigher mass might be observed because the IR samples were used for measuring mass spectra, which have been partially deuterium exchanged. See the section of *Isotope-Edited FTIR Spectroscopy*. 
Table S2. Peak fitting of the difference FTIR spectra of PenG covalent adducts

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<sup>a</sup>Gaussian-Lorentzian sum

<sup>b</sup>Ref. 4
Table S3. Largest field experienced by the linkage C=O in TEM–PenG

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\(^a\) The error of the field was calculated based on the error of probe calibration.\(^1\)

\(^b\) Ref. 4

\(^c\) Ref. 1
Figure S1. Positioning of the hydrolytic water in PBP-A and TEM-1. (A) Crystal structure of the PenG covalent adducts of PBP-A (PDB: 2J8Y)\textsuperscript{13}. PenG is only shown with the ester linkage for simplicity. PBP-A lacking a hydrolysis apparatus due to a nonpolar L158 residue. (B) Crystal structures of the PenG covalent adduct of PBP-A L158E (PDB: 2JBF)\textsuperscript{13} and TEM-1 E166N (PDB: 1FQG)\textsuperscript{14} overlayed by aligning their S61 and S70, respectively. The L158E mutation of PBP-A (yellow) adds a hydrolytic water (sphere) to an optimal position for attacking the ester linkage. The water in TEM-1 E166N deviates from that in PBP-A L158E by 1.4 Å, due to the E166N mutation necessary for trapping TEM–PenG. (C) Crystal structures of PBP-A (PDB: 2J9O)\textsuperscript{13} and TEM-1 (PDB: 7U6Q).\textsuperscript{1} The water superimposes better, deviating by 0.9 Å. The basic O atom in the E166 and L158E residues are perfectly superimposed.
Figure S2. Crystal structures of the A237Y mutant of TEM-1 show a potentially nucleophilic water positioned near to the backbone amide between G236 and A237Y. (A) Protein without Avb bonding (PDB: 8DDZ)\(^1\). The C=O of the backbone amide is engaged in two H-bonds, one with the backbone amide of G244, the other with the residue of R243. A water molecule’s O atom is positioned perpendicularly to the C=O bond axis, at a distance of 4.6 Å to the C atom in the C=O. A chain of water molecules is found between the nucleophilic water and the general base E166. (B) Protein covalently modified by Avb (PDB: 8DE1)\(^1\). The presence of Avb breaks the water chain.
Figure S3. HPLC-MS measures the fragmentation of the A237Yc mutants of TEM-1 due to the hydrolysis of the ester backbone. (A) HPLC chromatograph of the as-purified A237Yc. The peak at retention time 4.84 min corresponds to the fragment i of the protein. The peak at retention time 5.18 min corresponds to a mixture of the whole protein (wp) and the fragment ii. The two fragments were generated by the hydrolysis of the ester backbone. (B-D) Mass spectrum of the fragment i (B), the whole protein (C), and the fragment ii (D). (E-L) HPLC chromatograph of the A237Yc treated by 0.5 M NaOH for 10 min (E), the A237Yc left at pH 7 for 1d (F), 2d (G), and 6d (H), the as purified A237Yc E166N (I), the A237Yc E166N left at pH 7 for 6d (J), and the A237Yc reacted with 2 mM Avb and left at pH 7 for 1d (K) and 2d (L).
Figure S4. Progress of the hydrolysis of the ester backbone in the A237Yc mutants of TEM-1 at pH 7.0. The hydrolysis is slowed by forming a covalent complex with Avb or the E166N mutation. The yield is quantified by the peak area of the protein fragment $i$ in chromatographs obtained from HPLC-MS (Figure S3), using the NaOH-treated sample as the standard of 100% hydrolysis. Note that A237Yc E166N is not thermally stable enough for crystallization at RT. After being left at RT for two days, it cannot react with AVB any more as measured by MS. E166N is a general destabilizing mutation for TEM proteins.
Figure S5. $^{12}$C-$^{13}$C difference infrared absorption spectra for PenG covalent adduct of TEM-1 (trapped by E166N mutation) and PBP-A. (A) TEM-1 WT, (B) TEM-1 A237Y, (C) TEM-1 A237Y<sup>e</sup>, (D) TEM-1 A237E, (E) TEM-1 A237R, (F) TEM-1 A237W, (G) PBP-A. The experimental curve (black) is fitted to a sum (grey) of $^{12}$C (positive) and $^{13}$C (negative) peaks. Peaks belonging to the same positive-negative pair are filled with the same color.
Figure S6. IR absorption spectra displaying the vibrational peaks of the linkage C=O in the PenG covalent adduct of TEM-1 and PBP-A. The top electric field axis is mapped from the bottom frequency axis according to $\bar{\nu} = 0.68F + 1749.4$ as obtained in our previous work\textsuperscript{4}. $\bar{\nu}$ is the wavenumber (cm$^{-1}$) of the C=O vibrations, and $F$ is the magnitude of electric fields (MV/cm) projected on the C=O.
Figure S7. Zeroth-order hydrolysis of the carbamate linkage between TEM-1 and Avb. The linear regression line ($R^2 = 0.96$) gives a slope of $-8 \pm 1 \times 10^{-8} \text{s}^{-1}$.
**Figure S8.** Physical origins of ∆∆G‡. (A) Conceptual analysis of reactivity using the hydrolysis of an ester facilitated by two H-bonds as an example. Change in free energy barrier ∆∆G‡ can have an electrostatic origin, due to the change in field magnitude (∆∆G_F‡) or in reaction difference dipole (∆∆G_Δμrxn‡). ∆∆G‡ can also originate from the change in reactivities independent on environmental electric fields, including electrophilicity (∆∆G_E‡), nucleophilicity (∆∆G_Nu‡), and nucleophile positioning with respect to the electrophile (∆∆G_Pos‡). (B) An expansion of the range of fields of Figure 3A assigning the physical origins of ∆∆G‡ associated with ①–④.
**Figure S9.** Avb impairs deprotonation of the hydrolytic water compared with PenG in their covalent adduct with TEM-1. (A) Crystal structure of TEM$_{E166N}$–PenG (PDB: 1FQG)$^{14}$ and chemical illustration of TEM–PenG showing the two H-bonds of the hydrolytic water with E166 and N170. (B) Crystal structure of TEM–Avb (PDB: 7U6Q)$^1$ showing the three H-bonds of the hydrolytic water. The amide side group of the Avb and N170 together accepts two protons of the water, prohibiting the proton transfer to E166. The E166 likely exists in a protonated state, donating the third H-bond to the water.
Figure S10. MD simulation confirms a protonated E166 in TEM–Avb. (A) Representative snapshot of TEM–Avb with a protonated E166. The water forms a H-bond with the amide side group of Avb. (B) Representative snapshot of TEM–Avb with a deprotonated E166. The water cannot form a H-bond with the amide side group of AVB, leading to a wrong conformation of the amide side group of Avb. Although polarizable MD simulations were performed, we expect that a regular MD simulation\textsuperscript{15} can reproduce the same result.
Supplementary Text

Text S1. Intrinsic electrophilicity of carbamate and ester.

We analyzed the difference between the carbamate and ester bond with respect to their intrinsic reactivity towards hydrolysis using two model compounds. Ethyl acetate has a second-order rate constant of $1.1 \times 10^{-1}$ M$^{-1}$ s$^{-1}$ for hydrolysis via the base-catalyzed mechanism$^{16}$. The hydrolysis via the base-catalyzed mechanism is relevant because it involves a direct nucleophilic attack similar to enzymatic reactions, in contrast to a preceding protonation of the C=O in an acidic mechanism. The same rate constant for a simple carbamate, ethyl dimethyl carbamate, is $4.5 \times 10^{-6}$ M$^{-1}$ s$^{-1}$.$^{17}$ The $2.4 \times 10^{4}$-fold difference in reactivity can be converted to a gap in free energy barrier of $\Delta \Delta G_E^\ddagger = -5.9$ kcal/mol, with the ester having a lower free energy barrier than the carbamate.

This analysis is based on two assumptions. (1) The positioning of hydroxide as the nucleophile should be the same for the hydrolysis of ethyl acetate and that of ethyl dimethyl carbamate. This is a reasonable assumption because hydroxide should be similarly available in water for launching a nucleophilic attack. (2) The solvation sphere surrounding the C=O in ethyl acetate and that in ethyl dimethyl carbamate imposes a similar electrostatic effect. The IR spectra we collected previously showed that their C=O experiences similar electric fields in water: -67 MV/cm for ethyl acetate$^{18}$ and -75 MV/cm for a methyl piperidine-1-carboxylate,$^{1}$ another carbamate model compound.

Text S2. Motivation of analyzing the H-bond length.

In comparison of PBP-PenG and TEM-PenG, we note that the shortened H-bond is not the sole contributor of the 80-MV/cm change in electric fields, and that the distant charges/dipoles should also contribute, especially when they are well positioned. Our emphasis on H-bonds originated from the fact that the H-bonds are easier to perturb experimentally and to analyze directly from a chemical intuition, which was the motivation of this study.

We also note that the electric field can be very sensitive to the H-bond length. From 2.8 Å to 3.1 Å, the field magnitude can decrease significantly because the magnitude of the electric field is inversely proportional to the square of the distance between the charges. Please note that the 2.8 or 3.1 Å is the N-O distance in the N–H···O, not the shorter H-O distance which enters the equation. Although the crystal structure is not high enough resolution to provide the positions of the H atoms (protein structures rarely are), the 0.3 Å decrease in the N-O distance is expected to cause a significant shortening of the H-O distance. Other than the effect of the distance, a shorter H-bond also means more positive charge on the H atom and hence even larger electric fields projected by the H atom. There has been considerable work on this from simulations of simple solvents like water as well as Head-Gordon’s calculations related to our KSI results$^{19,20}$. Therefore, we identified the shortening of the H-bonds as a prominent aspect of protein evolution whose impact can be characterized using the vibrational Stark effect.
Text S3. Which contribute more to producing electric fields: Local or distant charges?

Computer simulations have suggested that the local H-bonds in the active site contribute more than the greater protein environment. Valerie Vaissier Welborn and Teresa Head-Gordon\textsuperscript{20} have shown that in the active site of ketosteroid isomerase, a model enzyme for the study of protein electrostatic catalysis, the three main active site residues contribute 90\% of the total electric field and are dominated by short H-bonds. The greater protein environment accounts for the remaining 10\%. Because this is not a simulation paper, we would like to leave the simulation to future studies. It is certainly interesting to use polarizable MD simulations to deconvolute the contributors to the electric fields in PBP-A and TEM-1, but this is an entire research program unto itself.

Text S4. Assumption: WT and L158E PBP–PenG have the same active-site electric fields.

For PBP–PenG, only the WT’s electric field magnitude can be measured because the L158E mutant’s linkage hydrolysis is not slow enough to maintain PBP–PenG over the time needed for IR measurements. We therefore make the simplifying assumption that the L158E mutation is non-perturbative to the electric fields experienced by the linkage C=O in PBP-A, in parallel with the previous observation that the fields in TEM–PenG remain unchanged going from E166N to E166A\textsuperscript{4} (E166 in TEM-1 corresponds to L158 in PBP-A with respect to position). This is reasonable because the PenG C=O bond axis points about 90\° from the direction of the nucleophilic L158E/E166. Therefore, we take the same value of the electric field magnitude measured for the WT PBP–PenG for analyzing L158E PBP–PenG.

Text S5. Assumption: Avb recyclization and hydrolysis have the same reaction difference dipole.

Although we measured the linkage hydrolysis rate of WT TEM–Avb, we cannot obtain the same rate for the A237Y\textsuperscript{c} mutant because the hydrolysis of the carbamate linkage is much slower than that of the backbone ester. To extrapolate $\Delta G^\ddagger$ to zero field, we borrowed the slope ($\Delta \mu_{\text{rxn}}$) from the recyclization reaction of TEM–Avb (1.4 D)\textsuperscript{1}, a reaction that starts with the same carbamate C=O and passes through a similar oxyanion intermediate.

Text S6. Physical origins of $\Delta \Delta G^\ddagger$

The physical basis for $\Delta \Delta G^\ddagger$ can be electrostatic (Figure S8A), where $\Delta \Delta G_F^\ddagger = -F \cdot \Delta \mu_{\text{rxn}}$ originating from a change in the magnitude of electric fields, while the reaction difference dipole $\Delta \mu_{\text{rxn}}$ remains the same. Conversely, when the electric field magnitude remains the same, a difference in reaction difference dipole produces $\Delta \Delta G_{\Delta \mu_{\text{rxn}}}^\ddagger = -F \cdot \Delta \mu_{\text{rxn}} \cdot \Delta \Delta G^\ddagger$ may also have a non-electrostatic origin (Figure S8A), including electrophilicity ($\Delta \Delta G_E^\ddagger$), nucleophilicity ($\Delta \Delta G_{\text{Nu}}^\ddagger$), and nucleophile positioning with respect to the electrophile ($\Delta \Delta G_{\text{Pos}}^\ddagger$).
We decompose the $\Delta \Delta G^\ddagger$ shown in Figure 3A (①–④) in terms of their different physical origins (Figure S8B). The $\Delta \Delta G^\ddagger$ contribution due to the L158E mutation of PBP-A (Figure 3A, ①) originates from the positioning and deprotonation of water, therefore falling into the category of $\Delta \Delta G_{\text{Nu}}^\ddagger + \Delta \Delta G_{\text{Pos}}^\ddagger$. The further evolution into TEM-1 (Figure 3A, ②) harnesses the power of increased electric fields, generating a $\Delta \Delta G_F^\ddagger$ down the slope of the linear correlation of electrostatic catalysis (Figure 3A, ②). Neither $\Delta \Delta G_{\text{μrxn}}^\ddagger$ or $\Delta \Delta G_E^\ddagger$ is involved in our analysis of protein evolution because both PBP-A and TEM-1 are connected to PenG by the same chemical bond.

The upgrade from Avb to PenG combines all the elements that contribute to $\Delta \Delta G^\ddagger$ (Figure S8B). The $\Delta \Delta G_{F=0}^\ddagger$ (Figure 3A, ③) can be split into $\Delta \Delta G_E^\ddagger$ and $\Delta \Delta G_{\text{Nu}}^\ddagger$. $\Delta \Delta G_E^\ddagger$ is calculated as 5.9 kcal/mol based on the lower electrophilicity of the carbamate linkage in TEM–Avb compared with that of the ester linkage in TEM–PenG (Text S1). The remaining 3.3 kcal/mol of $\Delta \Delta G_{F=0}^\ddagger$ is assigned to $\Delta \Delta G_{\text{Nu}}^\ddagger$, reflecting the poorer nucleophilicity of the hydrolytic water. In TEM–Avb, the hydrolytic water forms an extra H-bond to the amide side group of Avb (Figures S9,S10). Because the water is the H-bond donor, the proton is no longer available for the deprotonation by E166$^{21}$. The electrostatic component of $\Delta \Delta G^\ddagger$ (Figure 3A, ④) includes $\Delta \Delta G_F^\ddagger = 2.9$ kcal/mol and $\Delta \Delta G_{\text{μrxn}}^\ddagger = 2.0$ kcal/mol, calculated from the obtained $F$, $\Delta F$, $\Delta \mu_{\text{rxn}}$ and $\Delta \Delta \mu_{\text{rxn}}$ for WT TEM–Avb and TEM–PenG, and illustrated in Figure S8B.
References


