Engineering an Allosteric Binding Site for Aminoglycosides into TEM1-β-Lactamase


Allosteric regulation of enzyme activity is a remarkable property of many biological catalysts. Up till now, engineering an allosteric regulation into native, unregulated enzymes has been achieved by the creation of hybrid proteins in which a natural receptor, whose conformation is controlled by ligand binding, is inserted into an enzyme structure. Here, we describe a monomeric enzyme, TEM1-β-lactamase, that features an allosteric aminoglycoside binding site created de novo by directed-evolution methods. β-Lactamases are highly efficient enzymes involved in the resistance of bacteria against β-lactam antibiotics, such as penicillin. Aminoglycosides constitute another class of antibiotics that prevent bacterial protein synthesis, and are neither substrates nor ligands of the native β-lactamases. Here we show that the engineered enzyme is regulated by the binding of kanamycin and other aminoglycosides. Kinetic and structural analyses indicate that the activation mechanism involves expulsion of an inhibitor that binds to an additional, fortuitous site on the engineered protein. These analyses also led to the defining of conditions that allowed an aminoglycoside to be detected at low concentration.

Introduction

Enzymes, biological catalysts developed over the course of evolution, have a few fundamental characteristics in common: they transform their substrates with very high efficiency; most of them act specifically on a limited set of substrates; and a significant proportion of them are regulated by binding of ligands, "allosteric effectors", whose structures are different from those of their enzymes’ substrates. With few exceptions, natural allosteric enzymes are oligomeric assemblies that consist of several subunits. Binding of allosteric effectors appears to induce changes in the interactions between the subunits and, as a consequence, conformational changes in the region of the active site; this leads to an increase or a reduction in substrate affinity or catalytic activity. Various allosteric control mechanisms have been proposed or discovered,[1, 2] and a range of techniques has been used to discover allosteric communication pathways between active sites and regulatory sites within proteins.[3–5] A few monomeric enzymes have also been shown to be regulated by allosteric effectors. Natural effectors have been shown to activate a ribonucleotide reductase, and to modulate the specificity of a glucokinase, and their allosteric sites have been documented by crystallographic investigations.[6, 7]

Despite the continuing improvement in our understanding of the mechanisms of allostery, the engineering of allosteric regulation remains a challenge. De novo creation of allosteric binding sites was first achieved by the insertion of known epitopes of monoclonal antibodies into the loops bordering the active site. Binding of the antibodies inhibited or mildly activated the engineered enzymes.[8–10] This approach was used to develop novel bioanalytical tools.[11] A generalization of the strategy (that bypassed the requirement for information about the epitopes) was established later by introducing phage-displayed libraries of mimotopes (epitope mimics) into the surface loops, and by selecting enzymes that bound to monoclonal antibodies through these mimotopes.[12] Indirect activation could be brought about by competitive binding of the hapten to the cavity of the antibody, thus reversing its inhibitory effect.

However, this approach cannot be easily applied to engineer regulation by small molecules; in this case, activation by induction of a conformational change, or expulsion of an enzyme-bound inhibitor could prove to be more viable strategies. The former was achieved by the creation of libraries of fusion pro-
teins, in which β-lactamase was inserted randomly within the sequence of periplasmic maltose binding protein (MBP). Subsequent screening identified insertants whose activity could be switched on as a consequence of the natural conformational change induced in the MBP upon binding of its cognate ligand. Impressive activation factors were observed, and the activation itself was used for changing the nature of the activator (from maltose to sucrose) by a simple in vivo selection strategy. Conversely, when the haem binding domain of cytochrome b₅₆₂ was inserted randomly into the TEM1-β-lactamase, the activity could be inhibited or activated by haem binding.

The construction of small-molecule-activated inteins, that is, autosplicing proteins, was achieved by using similar strategies. The engineering of a light-dependent enzyme was carried out by insertion of a light-sensing signalling domain into a region of the enzyme that corresponded to an allosteric communication pathway. In all these cases, the activity-controlling device was a natural protein domain, whose conformation depended on an inducing event. These strategies have been extensively reviewed recently.

Here, we report the de novo engineering, by a directed evolution strategy, of an allosteric binding-site for aminoglycosides in a monomeric enzyme, TEM1-β-lactamase. β-Lactamases are enzymes involved in the resistance of bacteria against β-lactam antibiotics, which they hydrolyse very efficiently. TEM1-β-lactamase was chosen as it had been engineered for regulation by the strategies mentioned above, which would allow comparison, and because it has been reported to support a very robust reporter activity. Furthermore, compared to antibodies it is much easier to express in high yield. Aminoglycosides constitute another class of antibiotics that bind to the 30S subunit of bacterial ribosomes, and thus stall protein synthesis. Aminoglycosides have been used extensively by veterinary practitioners, and their presence in foodstuffs is a growing source of concern, and hence must be monitored.

Furthermore, as they are rather hydrophilic compounds, it is unlikely they would bind to the enzyme primarily by nonspecific hydrophobic interactions. We show that this enzyme is activated by the binding of aminoglycosides. We have solved the crystal structure of the free protein, and have performed a solution NMR study of its interaction with kanamycin. Overall, kinetic and structural analyses indicate that the activation mechanism involves expulsion of an inhibitor that binds to an additional, fortuitous site on the engineered enzyme.

Results

Selection of a kanamycin-binding mutant

A library of phage-displayed mutants of TEM1-β-lactamase was used in the selection experiments designed to find kanamycin-binding mutants. In this library, random peptide sequences had been inserted in loops between the N- and C-terminal helices and their adjacent β-strands, and three residues in a loop connecting two β-strands bordering the active site had been randomized. It was subjected to several rounds of biopanning on immobilized biotinylated kanamycin A (KanA, Scheme 1), then to screening for the effects of KanA on activity. Error-prone PCR was then applied to improve these effects.

Scheme 1. Structure of biotinylated kanamycin A used for the selection of KanA binding β-lactamase variants from libraries.

The selected KanA-binding enzyme (“BlaKr,” kanamycin-binding β-lactamase) was expressed, and purified from Escherichia coli.

BlaKr contains the following modifications when compared to the wild-type enzyme: a six-residue extension (RTSHRP) flanked by cysteines, which replaced G41–A42 in loop L2 (connecting the N-terminal helix to the first β-strand); an E240R mutation in the G238E240R241 loop bordering the active site; and Q269/R/A270K/T271K/M272T replacements in L1, the loop that connects the last β-strand to the C-terminal helix (Figure 1). An additional mutation, E104K, frequently found in extended-spectrum β-lactamases, was also selected. In this paper, we follow the residue-numbering scheme of Ambler et al. and use alphanumeric notation for the inserted residues (C41-R41a-T41b-S41c-H41d-R41e-P41f-C42). Analytical size-exclusion chromatography indicated that BlaKr exists in a single, monomeric form at the concentration used for the analysis (~10 μM), and does not undergo oligomerization or domain swapping, as frequently observed when loops are mutated.

Figure 1. Crystal structure of BlaKr. The engineered loops are highlighted; the E104K point mutation is shown as sticks; the S70 active site is space-filled. The amino acid sequences of the engineered loops are given below.

See page for the full sequence information.
Effect of ligands on BlaKr activity

The effect of KanA on BlaKr activity was initially monitored by following the decrease in UV absorbance upon benzylpenicillin ("PenG", penicillin G) hydrolysis at different KanA concentrations in MES (2-(N-morpholino)ethanesulfonic acid) buffer. A clear activation effect of KanA was observed (Figure 2). Experiments were then run at different pHs and in different buffers to better characterize the enzyme. A very puzzling observation was made: activation was observed only in aminosulfonic acid buffers. In order to clarify the activation mechanism, kinetic parameters were determined at various concentrations of MES and KanA in bis-tris buffer. Plots of $k_{cat}$, $K_m$ and $k_{cat}/K_m$ as a function of [KanA] at high MES concentration are shown in Figure 3A. The increased catalytic efficiency ($k_{cat}/K_m$) in the presence of KanA appears to result mainly from a decrease in $K_m$ with only a modest effect detected on $k_{cat}$. As shown in Figure 3B, in absence of KanA, $K_m$ increases linearly with [MES], which indicates that MES behaves as a competitive inhibitor. On the other hand, at saturation of KanA, $K_m$ varies only slightly. These observations are compatible with a mechanism in which KanA activates the enzyme by expulsing MES, as formalized in the following kinetic model (Scheme 2).

The experimental values of $k_{cat}$ and $K_m$ obtained at various concentrations of MES and KanA were used to extract the $k_{cat}^0$, $K_m^0$, $K_{cat}^{Kan}$, $K_m^{Kan}$, $K_{cat}^{Kan,E}$ and $K_m^{Kan,E}$ parameters (see the Experimental Section), where the “0” and “A” superscripts correspond to absence and saturation in aminoglycoside, respectively. (“A” stands for aminoglycoside or activator.) $K_{cat}^{Kan}$ and $K_m^{Kan}$ represent the affinity of KanA for the free enzyme and the enzyme–substrate complex, respectively (Table 1). Sim-
lar experiments, performed with the wild-type enzyme as a control, showed no significant inhibition by MES, and no activation by KanA.

The enzyme appears to have acquired two physically or functionally overlapping binding sites: a low affinity one for MES, and a high affinity one for KanA. Analogous kinetic analyses with various aminoglycosides (Scheme 3 A) and aminosulfonates (Scheme 3 B) documented the specificity of both binding sites (Tables 2 and 3). KanA was not the aminoglycoside ligand with the highest affinity. Kanamycin B and tobramycin, whose structures are similar, bound with approximately fivefold-higher affinity. Isepmicin and ribostamycin, whose structures are different, bound with slightly lower affinity. A comparison of the $K_m^{ap}$ or ($k_{cat}/K_m^{ap}$) values suggests that the last two aminoglycosides might be less efficient at expelling MES. The submicromolar affinities of the first three aminoglycosides are not very different from the affinities of monoclonal antibodies for kanamycin (20–40 nm), as calculated from the data of Watanabe or Loomans. Affinities for oligosaccharides or similar compounds are rarely in the nanomolar range, because the hydrogen bonds contributing to binding are solvated by water in the free protein and ligand.

As far as the aminosulfonic acids are concerned, BES is a better inhibitor than MES, which is in turn superior to taurine. MOPS, which contains an additional methylene group between the amine and the sulfonate moieties, appears to fit less tightly in its binding site (Table 3). Some aminosulfonate binding was still detected at saturation in KanA (the observed $K_m^{ap}$ does not extrapolate back to $K_m$, i.e., 0.09 mM); this indicates that the aminoglycosides are not fully competitive with the aminosulfonic acids. The ratio of binding constants ($K_m^{ap}$ versus $K_m^{b}$) is approximately ten except again for MOPS, which seems to be less efficiently expelled by KanA. Surprisingly, the low-affinity aminosulfonate binding-site appears to be more specific than the aminoglycoside site.

The effects of MES and BES on the hydrolysis of PenG and nitrocefin (a larger, chromogenic β-lactamase substrate) were also measured by following full-progress curves. The $K_m^{b}$ values obtained with nitrocefin were similar to those reported for PenG. However, the value of $k_{cat}/K_m$ extrapolated to saturation in MES does not tend to zero but to 26 ± 5%. By comparison, BES inhibited nitrocefin hydrolysis nearly quantitatively, while taurine at 0.1 mM hardly affected it at all. A small activity on PenG (~5%) remained in 0.1 mM MES, and none in 0.1 mM BES.

### Table 1. Kinetic parameters for the hydrolysis of PenG catalysed by BlaK in the presence of MES and KanA

<table>
<thead>
<tr>
<th>Aminoglycoside</th>
<th>$k_{cat}^{M}$ [s$^{-1}$]</th>
<th>$K_m^{ap}$ [mM]</th>
<th>($k_{cat}/K_m^{ap}$) [m$^{-1}$ s$^{-1}$]</th>
<th>$K_{ES}$ [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>isepamicin</td>
<td>1316 ± 357</td>
<td>0.28 ± 0.14</td>
<td>(6.6 ± 0.2) × 10$^4$</td>
<td>0.45 ± 0.11</td>
</tr>
<tr>
<td>kanamycin A</td>
<td>1481 ± 221</td>
<td>0.16 ± 0.08</td>
<td>(8.5 ± 0.7) × 10$^3$</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>kanamycin B</td>
<td>1328 ± 116</td>
<td>0.18 ± 0.03</td>
<td>(7.8 ± 0.6) × 10$^3$</td>
<td>0.045 ± 0.004</td>
</tr>
<tr>
<td>ribostamycin</td>
<td>1430 ± 210</td>
<td>0.26 ± 0.09</td>
<td>(6.2 ± 0.5) × 10$^3$</td>
<td>0.30 ± 0.08</td>
</tr>
<tr>
<td>tobramycin</td>
<td>1113 ± 68</td>
<td>0.14 ± 0.02</td>
<td>(8.7 ± 0.3) × 10$^4$</td>
<td>0.031 ± 0.011</td>
</tr>
</tbody>
</table>

### Table 3. Inhibition constants of aminosulfonic acids in bis-tris buffer (pH 6.3)

<table>
<thead>
<tr>
<th>Aminosulfonic acid</th>
<th>$K_i$ [mM]</th>
<th>$K_{i}^{KanA}$ [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>taurine</td>
<td>0.008 ± 0.004</td>
<td>0.06 ± 0.03</td>
</tr>
<tr>
<td>MES</td>
<td>0.003 ± 0.0004</td>
<td>0.023 ± 0.008</td>
</tr>
<tr>
<td>BES</td>
<td>0.0007 ± 0.0001</td>
<td>0.01 ± 0.0025</td>
</tr>
<tr>
<td>MOPS</td>
<td>0.013 ± 0.004</td>
<td>0.024 ± 0.005</td>
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</tbody>
</table>

### Scheme 3. Structures of the ligands binding to the enzyme BlaK, and tested in this work: A) aminoglycosides; B) aminosulfonic acids.
The effect of tobramycin on nitrocefin hydrolysis in 25 mM BES buffer started to be detected in the 100 nM concentration range (Figure 4) while BlaKr-specific activity increased more than 25-fold between 10 nM and 10 μM tobramycin. For food safety regulations, the maximum tolerated concentration of aminoglycosides in milk is of the order of 300 nM. For practical applications, however, additional work would be needed to eliminate potential interactions with other components in milk.

In addition to the ligands described above, negatively charged heparin fragments (disaccharide I-S, disaccharide II-H, disaccharide III-H), and glucosamine have been tested for their effect on BlaKr activity at concentrations ranging from 0 to 100 μM. None of them affected the activity.

![Figure 4](image-url)

**Figure 4.** Effect of increasing concentration of tobramycin on the hydrolysis of nitrocefin. Full hydrolysis curves of 5 μM nitrocefin in the presence of 20 nM BlaKr and various concentrations of tobramycin followed at 482 nm in BES (25 mM, pH 6.6, 25 °C). The kcat/Km parameters were obtained by dividing the derived first order rate constants kcat by the BlaKr concentration.

### Isotermal titration calorimetry (ITC)

ITC experiments were also performed, in order to assess MES and KanA binding to BlaKr by an independent method. Binding of phenethylibronic acid (PhBA), a transition-state analogue and competitive inhibitor of wild-type β-lactamase (wt Bla), was used as a reference. The binding parameters presented in Table 4 were obtained from titration curves that used a 1:1 binding model (see Figure S1 in the Supporting Information). The interaction stoichiometry appears to deviate significantly from unity. At this stage we do not have a satisfactory explanation for this observation. KanA binds to BlaKr in both MES and cacodylate buffer, while no binding is detected in phosphate buffer. A comparison of the apparent KanA binding constants to BlaKr or the BlaKr–PhBA complex in MES buffer (Kb = 24 and 41 μM, respectively) indicates that these two interactions are independent.

### Structural characterization

The crystal structure of free BlaKr was solved at 1.6 Å. The electron density was well defined along the protein chain, except for the C-terminal part of the molecules, where about 20 residues (including a His-tag) were not observed. Ramachandran plots show that more than 98% of the residues lie in favoured regions, and that all of them adopt an allowed conformation (for the refinement statistics see Table S2). The structure is similar to that of the wild-type protein, except for the loop insertion (Figure 1). The cysteines bordering the loop following the N-terminal helix form a disulfide bridge.

Despite numerous attempts to diffuse KanA into BlaKr crystals, and to co-crystallize BlaKr–KanA in different buffers, we have not succeeded so far in solving the structure of the complex. A likely cause of this failure is crystal packing of the individual protein molecules; this partially occludes the putative KanA binding site. In a structure obtained during the co-crystallization attempts, two sulfate ions were found, one of which interacts with the side chain of R240 and the NH atom of R241 in the engineered loops region where MES binds (see below).

For the detection of kanamycin binding by NMR spectroscopy, a 15N-[U-15C, U-15N] BlaKr sample was prepared. Its 2D 1H,15N TROSY spectrum showed good peak dispersion in both proton and nitrogen dimensions. The backbone resonance assignments, derived from a suite of 3D TROSY-type NMR experiments (Experimental Section), were facilitated by the published chemical shifts for the wild-type protein. In total, assignments were obtained for 96% of backbone 1H and 15N (for all non-proline residues), 98% of all 13Cα, 91% of all 13Cβ, and 91% of all 13C atoms. In addition to three residues (H26, S70, and A237), whose 1H/15N resonances were also unassigned in wt Bla, we could not assign the BlaKr backbone amides of T41b (part of the engineered loop), T272 (a point mutation), and five residues in K215–L221 stretch. Besides, weak peaks of S41c, M68, and R241—observed in the spectrum of BlaKr in sodium phosphate—disappeared in the spectrum acquired in MES. Overall, the BlaKr chemical shifts were in a good agreement with those for the wild-type protein except for the residues in and around the mutation sites. The chemical shifts are deposited in BioMagResBank (accession number BMRB 15917).

### Table 4. ITC binding parameters for BlaKr (0.7–1.5 mM) with different buffers (20 mM, pH 6.6, 30 °C).

<table>
<thead>
<tr>
<th>Interaction</th>
<th>Buffer</th>
<th>N [a]</th>
<th>Kd [μM]</th>
<th>ΔG° [kJ mol(^{-1})]</th>
<th>ΔH° [kJ mol(^{-1})]</th>
<th>ΔS° [kJ mol(^{-1})]</th>
<th>∆TΔS° [kJ mol(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>BlaKr + KanA</td>
<td>MES (4)</td>
<td>0.6 ± 0.1</td>
<td>24 ± 1</td>
<td>−26.9 ± 0.1</td>
<td>−9.6 ± 0.4</td>
<td>−17.1 ± 0.4</td>
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<tr>
<td></td>
<td>cacodylate (3)</td>
<td>0.8 ± 0.1</td>
<td>51 ± 2</td>
<td>−25.1 ± 0.1</td>
<td>−20.1 ± 2.1</td>
<td>−5.0 ± 2.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>phosphate (3)</td>
<td>n.d.</td>
<td>−0.1</td>
<td>−0.1</td>
<td>−0.1</td>
<td>−0.1</td>
<td></td>
</tr>
<tr>
<td>BlaKr + PhBA</td>
<td>MES (2)</td>
<td>0.7 ± 0.1</td>
<td>100 ± 6</td>
<td>−23.3 ± 0.2</td>
<td>−63.9 ± 6.7</td>
<td>40.6 ± 7.1</td>
<td></td>
</tr>
<tr>
<td>BlaKr-PhBA + KanA</td>
<td>MES (3)</td>
<td>0.6 ± 0.1</td>
<td>41 ± 3</td>
<td>−25.6 ± 0.2</td>
<td>−3.8 ± 1.3</td>
<td>−21.3 ± 1.3</td>
<td></td>
</tr>
</tbody>
</table>

[a] Binding stoichiometry. [b] Number of titrations performed. [c] Binding not detected.
In order to define the binding sites of KanA, 2D NMR spectra of $^{15}$N BlaKr in the presence of increasing amounts of KanA were acquired, and the binding-induced spectral changes analysed. In 20 mM sodium phosphate, the BlaKr spectrum remained unperturbed in the presence of up to fivefold excess of KanA (data not shown). When 20 mM MES was used instead of phosphate, addition of KanA led to chemical shift perturbations ($\Delta \delta$) of $^{15}$N and $^{13}$C nuclei for some BlaKr residues (Figure 5A). Throughout the titration, a single set of resonances was observed at each KanA concentration, the binding shifts were proportional to the amount of KanA added, and the line widths remained constant. This indicates that the BlaKr–KanA interaction is in fast exchange on the NMR chemical-shift timescale, with an estimated dissociation rate constant $k_{\text{off}} \geq 130 \text{s}^{-1}$. Changes in $\Delta \delta$ during the $^{15}$N BlaKr–KanA titration, monitored in a series of spectra, fit well to a 1:1 binding model, with a shared $K_d$ of 32 mM (Figure S2)—in agreement with the ITC-determined $K_d$ (24 $\mu$M). For a comparison with the value obtained in the kinetic experiments, these apparent constants should be divided by (1 + (MES)/$K_{\text{m}}$). The corrected value (3.6 $\mu$m) is ten times higher than that derived from the kinetics analysis. However, the NMR and ITC values were obtained at very different protein concentrations (millimolar vs nanomolar) and slightly different pH and temperature; this could account for the discrepancy.

The binding shifts observed upon KanA addition to BlaKr in MES were small: maximum values were $\Delta \delta_{N} = 0.1 \text{ ppm}$, $\Delta \delta_{C} = 0.3 \text{ ppm}$ and $\Delta \delta_{\text{av}} = 0.072$ for the weighted average, $\Delta \delta_{\text{av}} = \sqrt{(\Delta \delta_{N}^2 + \Delta \delta_{C}^2)/2}$. Several regions of BlaKr were affected, for example the loops adjoining the N- and C-terminal $\alpha$-helices and the areas encompassing residues T128–N132 and E166–D176 (Figure 5A). The chemical-shift perturbation map (Figure 5C) revealed a single binding patch on the surface of BlaKr, located around residues R41a–R41e (part of the engineered loop), I173–D176, and G267–K271 (mutations in the loop preceding the C-terminal helix). Interestingly, a number of buried amino acid residues—several of which sit close to or within the enzyme’s active site (e.g., N132 and E166)—also exhibit significant $\Delta \delta_{\text{av}}$ (Figure 5C).

Upon titration with PhBA, a number of $^{15}$N BlaKr resonances disappeared, and some reappeared elsewhere in spectra acquired at higher PhBA concentrations. Such behaviour indicates that the BlaKr–PhBA interaction is in slow exchange on the NMR chemical shift timescale; this necessitated the assignment of the backbone amide resonances of the bound form. Overall, the $\Delta \delta_{\text{av}}$ values for BlaKr–PhBA are much larger than those for BlaKr–KanA in MES, and the region affected by PhBA is different (Figure 5B). According to the chemical shift map (Figure S5D), PhBA docks to the substrate-binding pocket, and affects many residues in and around the enzyme’s active site (as expected for a transition state analogue).

Discussion

From a library of phage-displayed $\beta$-lactamase variants, we have isolated an enzyme (BlaKr) that features an allosteric binding site for aminoglycosides, as was expected from the designed selection protocol. Surprisingly, an additional low-affinity binding site for aminosulfonic acid was also detected in this enzyme. The evidence for aminoglycoside and aminosulfonic acid binding came from their effect on the kinetics of the hydrolysis of penicillin and cephalosporin substrates. Aminosulfonic acids inhibit the enzyme through an apparent competitive inhibition mechanism (effect on $K_{\text{m}}$); aminoglycosides appear to re-activate it by expulsion of the inhibitor. Conversely, the binding of aminosulfonic acids reduced the affinity of BlaKr for aminoglycosides. KanA binding was also observed by ITC except in phosphate buffer where no ITC signal was recorded, either because the $\Delta H_{\text{b}}$ is too small or because the affinity is much lower in this buffer. The absence of perturbation of the BlaKr spectrum by KanA-addition in phosphate buffer, and the high $K_{\text{v}}$ value (~1 mM) recorded by measurements of the effect of KanA on rates of PenG hydrolysis in the same buffer, support the second hypothesis. The occurrence of a number of arginine residues in the engineered site might explain this behaviour as the guanidinium function is known to interact strongly with phosphate anions.

BlaKr was nearly as active as TEM1-$\beta$-lactamase, which has been described as a perfect enzyme—that is, an enzyme whose activity is diffusion limited. Its $k_{\text{cat}}$ versus PenG was similar to that of the wild-type enzyme, and its $K_{\text{m}}$ was only slightly larger (~two- to threefold). The insertion and mutations in loops close to the active site did not significantly degrade the activity. As a consequence, BlaKr could barely be activated directly by an allosteric ligand. By comparison, the creation of a hybrid protein (by insertion of the TEM1-$\beta$-lactamase into the MBP$^{[13]}$ strongly deactivated the enzyme, and hence ligand-induced activation could be observed.

While BlaKr was extracted from a library in which random peptides had been inserted in two loops, an insertion was found only in loop L2 (Figure 1). The lack of any insertion in loop L3 might have resulted from its rare occurrence (~7%) in libraries selected for catalytic activity.$^{[24]}$

The backbone amide chemical-shift perturbations observed in the NMR spectra on addition of KanA to BlaKr in MES buffer (Figure 5A) could have arisen from KanA binding, MES expulsion or a combination of both. A plot of the difference between the BlaKr spectra in phosphate or MES buffer versus the perturbations induced by Kan-binding in MES sheds some light on this question (Figure 6A). Nine residues with $\Delta \delta_{\text{av, Kan}}$ larger than twice the standard deviation above their mean, had very similar $\Delta \delta_{\text{av, Kan}}$ and $\Delta \delta_{\text{av, PhBA}}=\Delta \delta_{\text{av, MES}}$ values (filled diamonds). This strongly suggests that these shifts result from an identical cause: MES expulsion by Kan versus “phosphate in–MES out” in the difference spectrum. Besides these nine points, only three additional amide signals (filled squares) featured significant $\Delta \delta_{\text{av, Kan}}$ shifts; they are also significant on the $\Delta \delta_{\text{av, PhBA}}=\Delta \delta_{\text{av, MES}}$ axis. This scarcity, and the generally low perturbations on the $\Delta \delta_{\text{av, Kan}}$ axis provide far too few indications to position the aminoglycosides binding site.

Based on the results of the extensive kinetics analysis and the limited NMR data presented in this work, we would like to propose that although MES behaves kinetically as a competitive inhibitor, it does not bind to the active site. It is likely that

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binding sites for aminosulfonic acids and substrates are contiguous but distinct, as shown by a comparison of the chemical-shift perturbations arising from KanA-binding/MES-displacement with those induced by interaction with the active site ligand, PhBA. The correlation coefficient of a plot of $\Delta \delta_{\text{av,PhBA}}$ versus $\Delta \delta_{\text{av,kan}}$ is close to zero (Figure 6B). However, MES binding induces a chemical shift in N132, a residue which interacts with the substrate side chain. Hence, MES might reduce the affinity of the enzyme for its substrate by indirect competition for an anchor point. The significant level of activity that remained at saturation in MES, at least with nitrocefin as substrate, supports this interpretation.

Two mechanisms can be proposed to explain the functional competition between the BlaKr ligands: steric interaction, and an indirect effect by induction of conformational changes. TEM1-β-lactamase has been described, on the basis of an NMR investigation, as one of the most ordered proteins, and the presence of a disulfide bridge in loop L2 is likely to increase
BlaKr rigidity. This would favour a mechanism of steric interaction. However, several observations suggest that this enzyme is responsive to binding events relatively far from the active site. In hybrid proteins, in which ligand binding domains were associated with the TEM-1-β-lactamase,\textsuperscript{11,14} conformational changes induced by binding were clearly transmitted to the enzyme and affected the activity. When variants of TEM1-β-lactamase (endowed with transition metal ions affinity) were selected from our insertant libraries, the binding of ions to histidine residues 15–20 from the active site activated or inhibited the enzyme.\textsuperscript{18} The binding of ligands to a serendipitous allosteric site, 16 Å from the active site characterized by X-ray crystallography, was shown to inhibit the enzyme by “core disruption”.\textsuperscript{19} All these observations are more consistent with an interpretation of allosteric regulation, in which the binding of a ligand affects the relative free energies of protein states that differ in their affinities for other ligands. Our observations fit better with this model. The size of the region perturbed by KanA-binding/ME5-displacement is clearly larger than the ligands themselves: distances of 30 Å are found between the Cα atoms of R41b and N132 or E166 (residues belonging to the SDN motif and the W-loop important for β-lactamase activi-

**Figure 6.** Chemical-shift maps of BlaKr in the presence of KanA or PhBA. Left pane: Δδ_{ph} in phosphate–Δδ_{av} in MES buffers plotted versus averaged chemical-shift perturbation on titration of BlaKr with KanA in MES. Right pane: Δδ_{av} plotted versus Δδ_{av} residues whose Δδ_{av} exceed (Δδ_{av}) by at least two σ are labelled and indicated by + (Δδ_{av}), Δ (Δδ_{av}<Δδ_{av}), and □ (both conditions). The continuous lines follow the linear correlation on all points; the parallel dotted lines are Δδ_{av} above and below. All experiments were performed in 20 mM buffers, pH 6.8, at 30 °C.

property of natural proteins, as shown by the results of recent high-throughput screening programs for drug discovery and crystallographic analyses that led to the serendipitous detection of many allosteric sites.\textsuperscript{41} Components of crystallization media are also frequently found by chance in protein cavities. We show also that a directed-evolution strategy can be used successfully to introduce an allosteric site onto an enzyme framework without impairing its activity. Interactions of the allosteric ligands with the enzyme (or among them) might lead to a modulation of function, that is, enzymatic activity. This suggests that there is an opportunity for designing molecular biosensors by engineering allosteric regulation functions. However, harnessing this property remains a challenge, and will require the design of more sophisticated selection protocols.

**Experimental Section**

**Materials:** Kanamycin A disulfate was purchased from Riedel-de Haën or Roche (NMR experiments). Ribostamycin sulfate, tobramycin, and heparin fragments (disaccharide I-S, disaccharide II-H, disaccharide III-H), phenethylboronic acid and the buffers (Biotechnology Performance Certified), MES, BES, MOPS and bis-tris were purchased from Sigma–Aldrich. Glucosamine hydrochloride, kanamycin B sulfate and taurine (Bioultra) were purchased from Fluka.

**Selection of BlaKr clone:** Biopanning experiments were performed in TBS (Tris buffer saline: 50 mM Tris.HCl, 150 mM NaCl, pH 7.5) on freshly prepared phages by using previously described phage libraries\textsuperscript{[7]} and protocols\textsuperscript{[12]} with minor modifications. The following supports were used: streptavidin Dynabeads M-270 (Dynal) and avidin-coupled agarose (Sigma), each presaturated for one hour with biotinylated kanamycin (30 μM in TBS containing 0.1% milk protein (M-TBS)). Two buffers were used to block nonspecific sites: M-TBS and G-TBS (TBS containing EIA grade gelatine (1% (w/v); BioRad). For the first two rounds of selection, elutions were performed by competition with 100 μM KanA; this was dialysed.
before infection of the bacterial culture by the eluted phages to prevent its inhibition. From the third round, elution was performed by tryptic cleavage (200 nm, 20 min) of the connector between the enzyme and the phage g3p protein. Elution yields after six rounds remained in the 10-3 range. DNA sequencing of 36 clones, picked randomly after six rounds of selection, pointed to consensus sequences, but ELISA tests did not give clear positive signals. Individual clones were screened to detect the effect of KanA on their hydrolytic activity on PenG. A phage–enzyme complex was found that showed a weak (~40%) increase in activity in phosphate buffer (apparent affinity \( K_d = 0.66 \pm 0.09 \) mm). This clone featured the following sequence in the loops: C41-R41a-T41b-S41c-H41d-R41e-P41f-C42, G238-R240-R241, R269-K270-K271. In view of its low KanA affinity, it was not studied in more detail, but it was subjected to a cycle of error-prone PCR, and the second-generation library was selected by five further rounds of biopanning on alternating supports as above. The number of washes was increased as selection progressed; elution was performed by competition with 100 \( \mu \)m KanA. Phages isolated by affinity selection were additionally selected for intracellular activity by spreading bacterial cells infected with selected phages on plates containing ampicillin and lysed by Microcal\textsuperscript{TM} Origin 5.0 with models included in the software package. In each case, the first data point was discarded, and kinetic parameters on the concentrations of aminoglycosides (e.g., KanA) or aminosulfonates (e.g., MES):

\[
K_{cat}^{obs} = \frac{K_{cat} \cdot K_{dES} + K_{cat}(\text{KanA})}{K_{dES} + [\text{KanA}]}
\]

\[
K_{dES}^{obs} = \frac{1 + \frac{[\text{MES}]}{K_a}}{1 + \frac{[\text{MES}]}{K_a}}
\]

\[
K_{cat}^{obs} = \frac{K_{cat} \cdot K_{dES} + K_{cat}(\text{KanA})}{K_{dES} + [\text{KanA}]}
\]

X-ray crystallography: Crystallizations of His-tagged proteins were performed at 18 °C using hanging drops. The first crystal form (BlaKr) was obtained in the absence of kanamycin. The reservoir contained 500 \( \mu \)L solution (bis-tris buffer (0.1 M, pH 6.2), PEG 6000 (25 % (w/v)), sodium azide (0.02 % (w/v)) and sodium chloride (0.3 M). In MES buffer (0.1 M, pH 6.5). PEG 5000 MME (30 % (w/v)) and ammonium sulfate (0.2 M). For the hanging drop, protein solution (0.5 M containing 1 mM kanamycin) was mixed with reservoir solution (0.5 M). Crystals (BlaKrSO\textsubscript{4}) appeared after several weeks. In both cases, for data collection the crystals were flash cooled (100 K). The addition of a cryo-protectant was not necessary. The data were processed by XDS and XS SCALE.\textsuperscript{[24]} Both structures are orthorhombic, space group P2\textsubscript{1}, 2\textsubscript{1}, 2\textsubscript{1}, with one molecule in the asymmetric unit (for data analysis, see the Supporting Information). The final coordinates and B-factors were deposited in the Protein Data Bank (IDs: 2v1z and 2v20). The residue numbering in the deposited structures is different from the conventional Ambler numbering\textsuperscript{[20]} (conversion in Table S3).

**Preparation of BlaKr samples:** The gene coding for BlaKr was cloned into a PET24(ompA) expression plasmid\textsuperscript{[17,18] downstream of the OmpA signal peptide, and into a vector derived from pBAD/Myc-His (Invitrogen) to express the protein either extracellularly or intracellularly, free or in fusion with a His-tag, respectively. It was purified by following published procedures.\textsuperscript{[14,42]} Uniformly labelled \(^{15}\)N and \(^{13}\)C proteins were produced in M9 minimal medium with \(^{15}\)NH\textsubscript{4}Cl (99% pure, Cambridge Isotope Laboratories) and \(^{13}\)C\textsubscript{D} glucose (99% pure, CorteNet, Paris) as sole nitrogen and sugar sources. Protein concentrations were determined from UV/Vis spectra by using a calculated\textsuperscript{[21]} value of the extinction coefficient, \( e_{280} = 28.21 \) M\textsuperscript{-1} cm\textsuperscript{-1}.

**Kinetics:** BlaKr-catalysed hydrolysis was followed by the decrease in UV absorbance of the PenG substrate at 232 nm (\( \lambda = 1040 \) nm\textsuperscript{-1} cm\textsuperscript{-1}) or, for solutions containing bis-tris (25 mm) at 239 nm (\( \lambda = 689 \) nm\textsuperscript{-1} cm\textsuperscript{-1}). PenG (100 \( \mu \)L) and aminoglycoside (10 \( \mu \)L) were added to buffer (2 mL, aminosulfonic acids or bis-tris buffer at various concentrations) and equilibrated at 25°C. Then stock enzyme solutions (10 \( \mu \)L, from 500 nm to 1 \( \mu \)L, containing 10 \( \mu \)L BSA) were added. The \( K_{cat}^{app} \) and \( K_{m}^{app} \) parameters were calculated from the initial rate constants \( k_{cat}^{app} \) measured at several substrate concentrations; they were estimated to within 10% and 20% fluctuations, respectively, in independent experiments. These parameters were plotted as functions of aminoglycoside concentration, and the \( K_{cat}^{app} \), \( k_{cat}^{app} \) and \( K_{m}^{app} \) values were determined by nonlinear regression on \( K_{m}^{app} \) and \( K_{m}^{app} \) weighted by their 95% confidence limits. For the KanA–MES system, the true parameters \( K_{m}^{app}, K_{m}^{app}, K_{m}^{app}/K_{m}^{app}, K_{m}^{app}/K_{m}^{app}, K_{m}^{app} \) and \( K_{m}^{app} \) (as defined in Scheme 1 and Table S1) were obtained by fitting 72 \( k_{cat}^{app} \) and \( K_{m}^{app} \) value pairs obtained at various concentrations of these ligands. As the influence of the aminoglycoside concentration on \( k_{cat}^{app} \) is small, these parameters were not used to determine \( K_{cat}^{app} \).

Myc labels containing 1 m\textsuperscript{38}S were purified by following published procedures.\textsuperscript{[24]} Additions of a cryo-protectant were not necessary. The data were collected at 4°C. For backbone amide assignments of the free and PhBA-bound forms, two samples containing [U-\(^{13}\)C, U-\(^{15}\)N] BlaKr (0.6 M in sodium phosphate (20 mm, pH 6.6)), with and without five molar equivalents of PhBA, were used. To transfer the assignments to the resonances of BlaKr in MES, 2D \(^{1}H,^{1}H\) TROSY spectra were acquired of [\(^{15}\)N BlaKr (0.4 M in MES (20 mm, pH 6.6)). In the presence of


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sodium phosphate (0, 10, and 20 mM). For the NMR binding studies, 15N BlaKr (0.3–0.5 mM in sodium phosphate (20 mM, pH 6.60) or MES (20 mM, pH 6.60)) was titrated with concentrated stock solutions of Kan or PhBA (10–20 mM) in the same buffer. The pH of the samples was checked during the titrations and, if necessary, adjusted to 6.60 ± 0.05 with 0.1 M HCl or NaOH. Measurements were performed at 30 °C on a DMX 600 MHz or Avance 900 MHz spectrometer (Bruker Corporation, Billerica, MA), equipped with TCI-Z-GRAD or TCI 9005E H/C/N-o-05 Z cryoprobes (Bruker), respectively. The binding of KanA and PhBA was followed in a series of 2D 1H,15N TROSY-HSQC spectra.[45] For further details, see the Supporting Information.

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**Evolving regulation:** An allostERIC binding site for aminoglycoside antibiotics has been created in TEM1-β-lactamase by directed evolution. Aminoglycosides binding activates the enzyme by expulsion of an inhibitor, and allows their detection at low concentration. Interactions with several ligands offer opportunities for complex allostERIC regulation.