

# Mixed-type noncompetitive inhibition of anthrax lethal factor protease by aminoglycosides

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## Keywords

aminoglycosides; *Bacillus anthracis*; inhibition; lethal factor protease; mechanism

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We report a detailed kinetic investigation of the aminoglycosides neomycin B and neamine as inhibitors of the lethal factor protease from *Bacillus anthracis*. Both inhibitors display a mixed-type, noncompetitive kinetic pattern, which suggests the existence of multiple enzyme–inhibitor binding sites or the involvement of multiple structural binding modes at the same site. Quantitative analysis of the ionic strength effects by using the Debye–Hückel model revealed that the average interionic distance at the point of enzyme–inhibitor attachment is likely to be extremely short, which suggests specific, rather than nonspecific, binding. Only one ion pair seems to be involved in the binding process, which suggests the presence of a single binding site. Combining the results of our substrate competition studies with the ionic strength effects on the apparent inhibition constant, we propose that aminoglycoside inhibitors, such as neomycin B, bind to the lethal factor protease from *B. anthracis* in two different structural orientations. These results have important implications for the rational design of lethal factor protease inhibitors as possible therapeutic agents against anthrax. The strategies and methods we describe are general and can be employed to investigate in depth the mechanism of inhibition by other bioactive compounds.

The lethal factor protease from *Bacillus anthracis* is the dominant virulence factor in anthrax infection [1]. For this reason, inhibitors of the protease are being sought as possible therapeutic agents. Several types of small polycationic molecules have been identified as selective and potent lethal factor inhibitors. For example, Lee *et al.* [2] screened a diverse library of natural and synthetic compounds *in vitro* and discovered that polycationic aminoglycosides, such as neomycin B, are very potent inhibitors. In a follow-up study *in vivo*, Fridman *et al.* [3] demonstrated that neomycin B and other aminoglycosides have an antibacterial effect.

These authors [2], as well as we [4] and others [5], postulated that one of the main structural reasons why polycationic inhibitors bind strongly to the lethal

factor protease is electrostatic attraction between the inhibitors and a patch of negative charges on the enzyme surface. This hypothesis was based on the microscopic X-ray structure of the enzyme active site [2,5] and on the macroscopic effects of ionic strength on the apparent inhibition constant [3].

Several important questions remain unanswered about the molecular details governing the inhibition of the lethal factor protease by aminoglycosides. For example, the kinetic mechanism of inhibition by neomycin B has been reported as being competitive with the substrate [3]. However, our data show that neomycin and other aminoglycosides clearly deviate from the competitive kinetic pattern. Reliably determining the kinetic mechanism of inhibition is important,

## Abbreviations

AIC, Akaike information criterion;  $d$ , effective interionic distance;  $[E]$ , enzyme active-site concentration; FRET, fluorescence resonance energy transfer;  $[I]$ , inhibitor concentration;  $K_i^{(app)}$ , apparent inhibition constant;  $K_i$ , competitive inhibition constant;  $K_{is}$ , inhibition constant; MAPKKide, mitogen-activated kinase kinase;  $[S]$ , substrate concentration.

because such kinetic measurements provide important insights into the structural binding mode [6].

Another question concerns the exact nature of electrostatic interactions between the enzyme and inhibitor molecules. Fridman *et al.* [3] measured the apparent inhibition constant for neomycin B at two different sodium chloride concentrations, but the detailed nature of these ionic strength effects on the strength of inhibition binding was not elucidated. In previous studies, we [7] and others [8] demonstrated that a quantitative analysis of ionic strength effects was able to distinguish between short-range specific electrostatic interactions and long-range nonspecific electrostatic interactions. Given the presence of multiple electrostatic charges on the protease and on the aminoglycoside inhibitors, it seemed important to assess the specificity in inhibitor binding using a similar method.

The purpose of this study was twofold. First, we wished to elucidate the kinetic mechanism of inhibition by which neomycin B and other aminoglycosides interact with the protease enzyme. If these inhibitors were strictly kinetically competitive with the protease substrate, the results would strongly support the simple binding model previously described in the literature [2,5]. According to this structural model, each positively charged inhibitor molecule attaches directly to the negatively charged active site on the enzyme. However, in our own preliminary studies we found that neomycin B is not strictly competitive with the substrate. This suggests that the structural binding mode is more complex than previously believed. Our goal was to explain the discrepancy between the published results, which suggest that neomycin B is a competitive inhibitor, and our own preliminary results, which suggest otherwise. The results reported here show that a plausible explanation of this discrepancy relies on properly accounting for substrate inhibition, rather than assuming that the peptide substrate follows the Michaelis–Menten kinetic model. Second, we set out to determine the dependence of the apparent inhibition constant,  $K_i^{(app)}$  [9], on the ionic strength of the buffer over a wide range of sodium chloride concentrations. The results were analyzed quantitatively using the electrostatic binding model [7,8], with the goal of determining the effective charge on the enzyme active site and the average interionic distance at the point of initial attachment of the inhibitor. We found that, unlike in the previously studied cases [7,8], the average interionic distance between the enzyme and the inhibitor at the point of initial contact is probably extremely short. In conjunction with the fact that neomycin B is not kinetically competitive with the peptide substrate, we propose that the aminoglycoside inhibitors attach to

their specific binding sites in at least two different kinetically competent structural orientations.

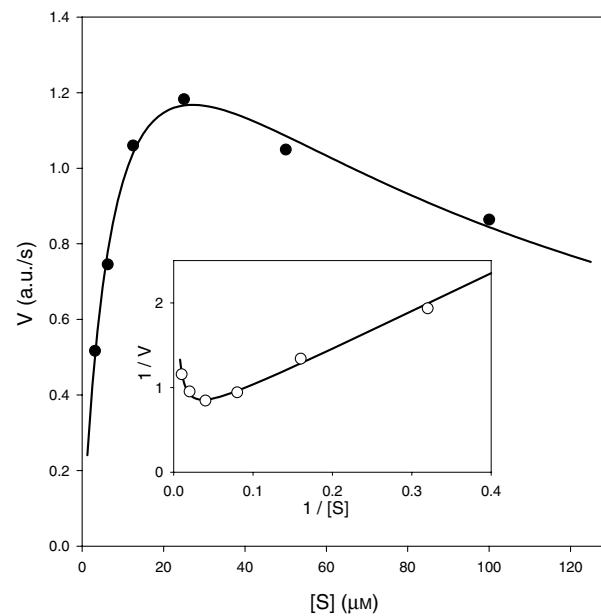
## Results

### Substrate kinetics

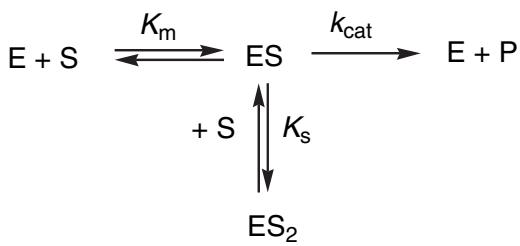
In order to determine reliably the kinetic mechanism of inhibition, it was necessary to characterize independently the unusual substrate kinetics of peptide substrate. The substrate saturation curve shown in Fig. 1 has a distinct maximum, which demonstrates that the conventional Michaelis–Menten model for substrate kinetics is not applicable. The experimental data in Fig. 1 were fit to the kinetic mechanism shown in Scheme 1. The corresponding mathematical model was generated automatically by using the software DYNAFIT, under the rapid-equilibrium approximation. Details of the automatic model derivation have been described previously [10]. The Michaelis constant,  $K_m$ , was  $8.6 \pm 1.5 \mu\text{M}$ , and the substrate inhibition constant was  $85 \pm 17 \mu\text{M}$ .

### Determination of inhibition mechanisms

Each model discrimination experiment was performed in two stages to optimize the experimental design. In



**Fig. 1.** Substrate inhibition of the lethal factor (LF) protease. The LF protease (13 nM) was assayed using the fluorogenic substrate, as described in the Experimental procedures. The experimental data (filled circles) were fit to the theoretical model represented by Scheme 1, using the software DYNAFIT [11]. The best fit values of kinetic constants appearing in the mechanism were  $K_m = 8.6 \pm 1.5 \mu\text{M}$  and  $K_s = 85 \pm 17 \mu\text{M}$ .

**Scheme 1.** Substrate inhibition mechanism.

the first stage, the  $K_i^{(app)}$  for each inhibitor was determined in a preliminary series of experiments, conducted at a single substrate concentration ( $12.5 \mu\text{M}$ , data not shown). The inhibition constants were determined by a least-squares fit to Eqn (1):

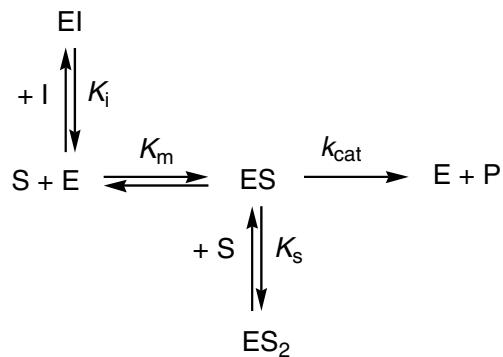
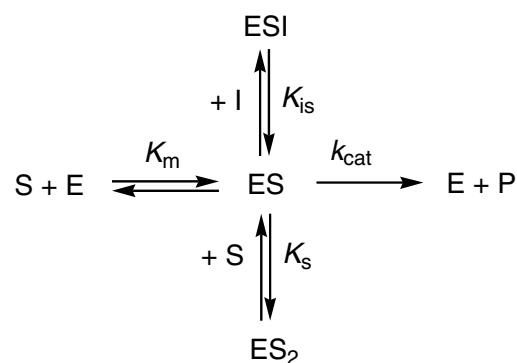
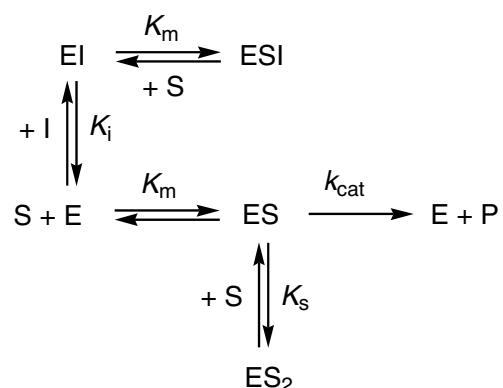
$$v_0 = V_b + V_0 \times \frac{[E] - [I] - K_i^{(app)} + \sqrt{([E] - [I] - K_i^{(app)})^2 + 4[E]K_i^{(app)}}}{2[E]} \quad (1)$$

where  $[E]$  represents the enzyme active-site concentration,  $v_0$  is the initial reaction rate observed at the inhibitor concentration  $[I]$ ,  $V_b$  is a baseline initial rate, and  $V_0$  is the initial rate observed at  $[I] = 0$ . (*AIC*, second order Akaike information criterion). Subsequently, three different inhibitor concentrations ( $[I]$ ) were chosen such that they were equal to  $[I] = 0.75 \times K_i^{(app)}$ ,  $[I] = 1.50 \times K_i^{(app)}$  and  $[I] = 3.00 \times K_i^{(app)}$ . At those particular inhibitor concentrations, and in a control series of experiments at  $[I] = 0$ , the substrate concentration ( $[S]$ ) was varied in a linear dilution series starting at  $10 \mu\text{M}$  and stepping by  $10 \mu\text{M}$  increments ( $[S] = 10, 20, 30, \dots, 70, 80 \mu\text{M}$ ). In a series of preliminary heuristic simulations, we established that this linear dilution series has a higher model-discrimination power than the conventionally used logarithmic series (e.g.  $[S] = 80, 40, 20, 10, 5, 2.5, 1.25 \mu\text{M}$ ). The  $8 \times 4 = 32$  combinations of  $[S]$  and  $[I]$  were used, in triplicate, to fill a 96-well plate. Initial reaction velocities ( $v_0$ ) in each well were determined by the nonlinear fit to Eqn (2):

$$F(t) = F_0 + F_1 \exp(-kt) \quad (2)$$

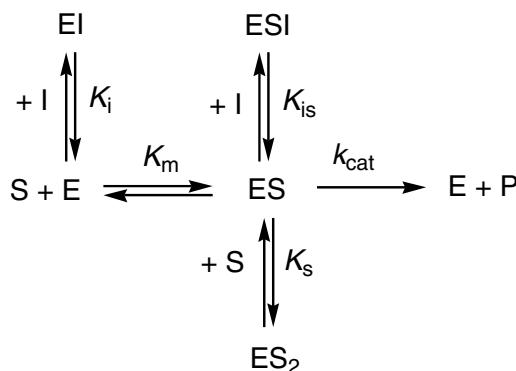
where  $F(t)$  is the fluorescent signal observed at time  $t$ ,  $F_0$  is the baseline offset,  $F_1$  is the exponential amplitude, and  $k$  is the first-order rate constant. The average from each group of three replicated initial rates was used in the model discrimination analysis. The typical coefficient of variation within each replicate was between 3 and 5%.

For each inhibitor, the matrix of 32 averaged initial velocity data points was analyzed by DYNAFIT [11]

**Scheme 2.** Competitive inhibition mechanism.**Scheme 3.** Uncompetitive inhibition mechanism.**Scheme 4.** Noncompetitive inhibition mechanism.

while considering four alternate mechanisms shown in Scheme(s) 2–5.

Initial reaction rates were fit to four alternate kinetic models (competitive, uncompetitive, noncompetitive and mixed-type) while taking into account the possibility of ‘tight-binding’ [19]. The mathematical models for each mechanism were generated, under the rapid-equilibrium approximation, as systems of simultaneous nonlinear algebraic equations solved by the multidimensional

**Scheme 5.** Mixed-type mechanism.

dimensional Newton–Raphson method. Details have been described previously [10]. The model discrimination analysis employed the second-order  $AIC_c$ , as defined by Eqn (6) ([12], p. 66). In Eqn (6),  $n$  represents the number of experimental data points (initial velocities),  $v_i$  is the  $i$ th experimentally determined initial rate,  $\hat{v}_i$  is the corresponding theoretical best-fit model rate computed by DYNAFIT [11] and  $K$  is the number of adjustable parameters:

$$AIC_c = n \log \left( \frac{1}{n} \sum_{i=1}^n (v_i - \hat{v}_i)^2 \right) + 2K + \frac{2K(K+1)}{n-K-1} \quad (3)$$

The AIC difference for the  $i$ th model being evaluated for plausibility among  $R$  alternate models is defined by Eqn (1), in which  $AIC_c^{(\min)}$  is the lowest second-order AIC found among the alternatives ([12], p. 71). The Akaike weights,  $w_i$ , for each model are defined by Eqn (7) ([12], p. 75). The model with the highest Akaike weight (maximum possible value 1.0) is considered the most plausible model among the alternatives under consideration:

$$\Delta_i = AIC_c^i - AIC_c^- \quad (4)$$

$$w_i = \frac{\exp(-\frac{1}{2}\Delta_i)}{\sum_{r=1}^R \exp(-\frac{1}{2}\Delta_r)} \quad (5)$$

The results are summarized in Table 1.

To decide on the plausibility of each candidate mechanistic model, we used the heuristic criteria devised by Burnham & Anderson ([12], p. 70). In particular, if a given kinetic mechanism is characterized by the AIC difference  $\Delta_i > 10$ , the plausibility of this model presumably is ‘essentially zero’. Burnham & Anderson further ascribe ‘considerably less’ (but not zero) plausibility for models characterized by AIC dif-

**Table 1.** Model discrimination analysis for inhibitors of the lethal factor protease. The competitive, uncompetitive, noncompetitive and mixed-type mechanisms are shown in Scheme(s) 2–5, respectively.  $K$  is the number of adjustable model parameters in each model. The Akaike information criterion ( $AIC$ ) differences and Akaike weights are defined in Eqns (4) and (5), respectively.

Mechanism	K	AIC difference, $\Delta_i$		Akaike weight, $w_i$	
		Neomycin	Neamine	Neomycin	Neamine
Competitive	4	5.9	15.9	0.049	0.011
Uncompetitive	4	80.6	35.1	0	0
Noncompetitive	4	27.5	9.0	0	0
Mixed type	5	0	0	0.951	0.989

ferences between 4 and 7 and, finally, models with  $\Delta_i < 2$  are considered to be all equally plausible.

In light of the heuristic rules of Burnham & Anderson, the most plausible inhibition mechanism for both inhibitors was mixed-type noncompetitive. However, Table 1 also shows that in the case of neomycin B the competitive mechanism (characterized by  $\Delta_i = 6$ ) perhaps represents a borderline case. Therefore, we have applied an additional test for statistical model discrimination according to the nested-model method described by Mannervik [13].

According to this method, a significance ratio for two nested models is computed as  $F = (S_1 - S_2)/S_2 \times (n-p_1)/(p_2-p_1)$ . Here,  $S_1$  and  $S_2$  are the two residual sums of squares,  $p_1$  and  $p_2$  are the corresponding number of adjustable model parameters and  $n$  is the number of experimental data points. The computed  $F$  ratio is then compared with the Fisher’s  $F$  statistic at the given significance level  $\alpha$ ,  $F_\alpha(n-p_1, p_2-p_1)$ . In the case of neomycin B, the competitive mechanism gave the sum of squares  $S_1 = 0.000343$  with  $p_1 =$  four adjustable model parameters. The mixed-type noncompetitive mechanism gave the sum of squares  $S_2 = 0.000259$  with  $p_1 =$  five adjustable model parameters. With 32 data points ( $n = 32$ ), the resulting ratio  $F = 9.0$  is higher than the critical value of Fisher’s  $F$  at the 99% confidence level,  $F_{0.005}(n-p_1, p_2-p_1) = 7.6$ . Thus, the mixed-type noncompetitive model should be considered more plausible than the competitive model.

**Table 2.** Best-fit values of inhibition constants in the mixed-type kinetic model and the corresponding 95% confidence intervals (CI).

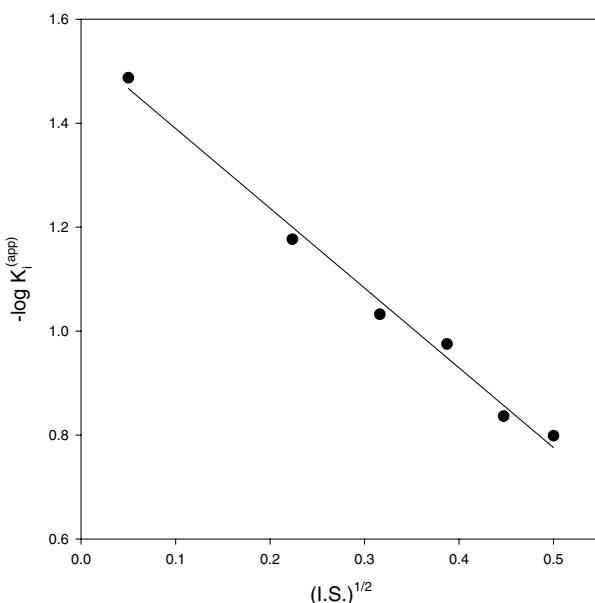
Inhibitor	$K_i$ ( $\mu M$ )	$K_i$ (95% CI)	$K_{is}$ ( $\mu M$ )	$K_{is}$ (95% CI)	$K_{is} : K_i$
Neomycin B	0.28	0.22–0.36	3.2	1.8–10.1	11
Neamine	13	8–22	0.064	41–114	5

The final test of plausibility of the mixed-type non-competitive model relied on determining the confidence interval for the inhibition constant  $K_{is}$  appearing in Scheme 5. The 95% confidence intervals for inhibition constants appearing in the mixed-type mechanism are summarized for both inhibitors in Table 2. In the case of neomycin B, the 95% confidence interval for  $K_{is}$  ranged from 1.8 to 10.1  $\mu\text{M}$  (with a best-fit value of 3.2  $\mu\text{M}$ ).  $K_{is}$  is well determined by the experimental data, which lends support to the mixed-type mechanism as the most plausible alternative among the four candidate mechanistic models.

The same conclusions were reached for neomycin B and neamine. Both compounds are mixed-type non-competitive inhibitors of lethal factor.

### Ionic strength effects

The  $K_i^{(\text{app})}$  for neomycin B was determined at six different concentrations of sodium chloride in the buffer; the results are shown in Fig. 2. We originally intended to use the Debye–Hueckel equation (Eqn 6) as the standard electrostatic binding model:



**Fig. 2.** Inhibition of the lethal factor (LF) protease by neomycin B: ionic strength ( $I.S.$ ) effects on the apparent inhibition constant. The apparent inhibition constants  $K_i^{(\text{app})}$  were determined by nonlinear least-squares fit of initial rates, observed at various concentrations of sodium chloride in the buffer, to Eqn (1). The best-fit values of  $K_i^{(\text{app})}$  were fit to Eqn (7) to determine the effective charges. The best-fit value of the slope parameter  $1.18 \times Z_E \times Z_L$  is 1.54, from which  $Z_E \times Z_L \approx 1.3$ , suggesting that only a single ionic pair is involved in inhibitor binding.

$$\log K_i^{(\text{app})} = \log K^* + \frac{1.18Z_EZ_L\sqrt{I}}{1 + 0.329d\sqrt{I}} \quad (6)$$

in which  $I$  is the ionic strength of the buffer,  $Z_E$  is the effective electrical charge on the enzyme molecule,  $Z_L$  is the effective electrical charge on the inhibitor and  $d$  is the average interionic distance.

However, preliminary analyses suggested that the best-fit value of the effective interionic distance ( $d$ ) was indistinguishable from zero. In fact, the best theoretical model for the available data is Eqn (7), representing a straight line, in which  $d = 0$  by definition. This result suggests that the distance between the inhibitor and enzyme molecules is extremely short, corresponding to specific binding, rather than nonspecific long-range electrostatic interactions. The slope of the best-fit line in Eqn (7) is  $-1.53$ , from which we can calculate the product of effective charges as  $Z_EZ_L = -1.3$ . This result suggests that, effectively, a single ion pair is probably responsible for the bulk of the enzyme–inhibitor binding interaction.

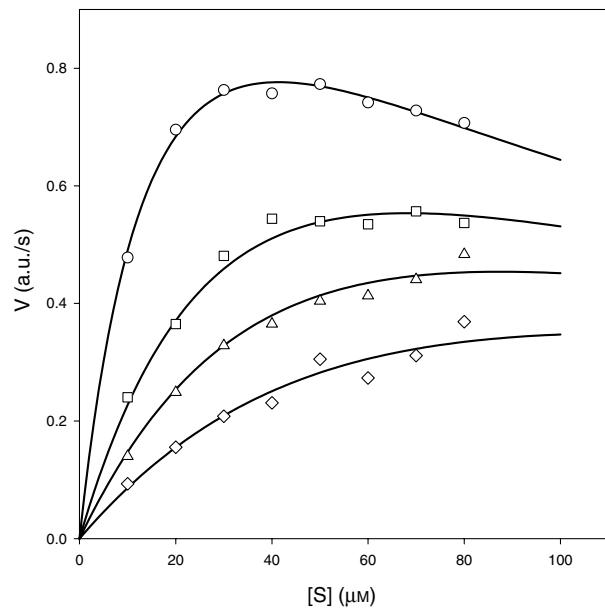
$$\log K_i^{(\text{app})} = \log K^* + 1.18Z_EZ_L\sqrt{I} \quad (7)$$

### Discussion

In this study we have determined that neomycin B and its close structural analog, neamine, are mixed-type noncompetitive inhibitors of the lethal factor protease from *B. anthracis*. This finding contradicts recent reports in the literature [3], where it is suggested that neomycin B is purely a competitive inhibitor. The difference between the two mechanisms has important implications for the rational design of lethal factor inhibitors as potential therapeutic agents. For example, a kinetically competitive inhibitor can always be displaced from the enzyme active site by a sufficiently high local concentration of the native substrate. In contrast, the effectiveness of a noncompetitive inhibitor is not at all sensitive to the substrate concentration.

In the following discussion we offer a possible explanation for the discrepancy between our results and those reported in earlier literature, and suggest an appropriate experimental design for reliable determination of inhibition mechanisms.

Fridman *et al.* [3], in their study, used an unspecified fluorescent substrate, one of several fluorogenic peptides previously described by Turk *et al.* [5]. Importantly, these authors used only four distinct substrate concentrations; inhibition constants and the (competitive) inhibition mechanism itself were ‘estimated from double reciprocal plots’. To reproduce this particular

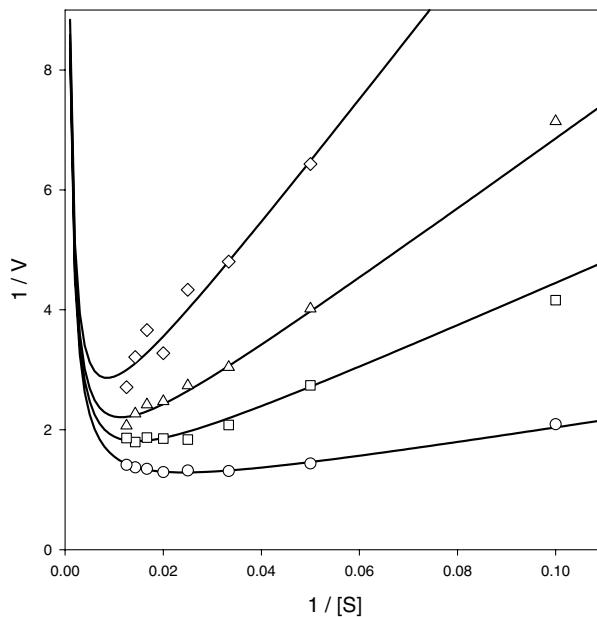


**Fig. 3.** Inhibition of the lethal factor (LF) protease by neomycin B: least-squares fit of the complete data set to the mixed-type model. The initial rates from assays of the LF protease (13 nM) were determined at various concentrations of the substrate ( $[S] = 10, 20, 30, \dots, 70, 80 \mu\text{M}$ ) and neomycin as the inhibitor ( $(\circ)$ ,  $[I] = 0$ ;  $(\square)$ ,  $[I] = 0.5 \mu\text{M}$ ;  $(\triangle)$ ,  $[I] = 1.0 \mu\text{M}$ ;  $(\diamond)$ ,  $[I] = 2.0 \mu\text{M}$ ). The theoretical curves were generated by least-squares fit to the mixed-type noncompetitive inhibition model represented by Scheme 5. The underlying mathematical model was automatically derived by DYNAFIT [11] under the rapid-equilibrium approximation [10]. The best-fit values of inhibition constants  $K_i$  and  $K_{is}$  are summarized in the first row of Table 2.

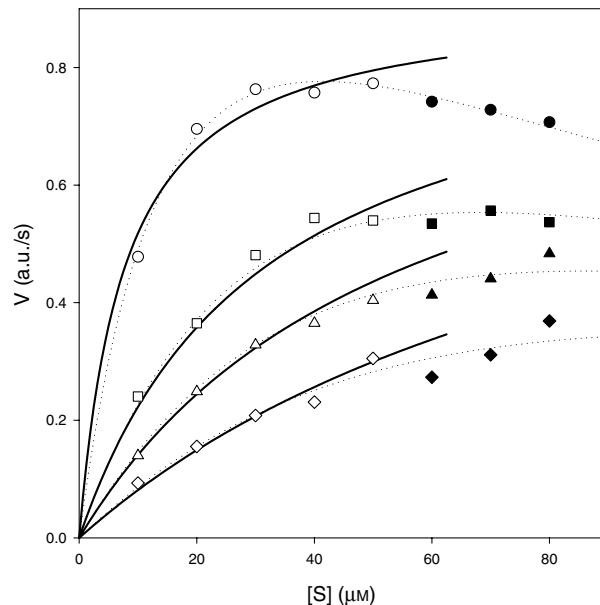
experimental design, we analyzed a subset of our experimental data, taking into account five relatively low  $[S]$  values (10, 20, 30, 40 and 50  $\mu\text{M}$ ). Importantly, we ignored the three highest  $[S]$  values (60, 70 and 80  $\mu\text{M}$ ) at which substrate inhibition is clearly manifested in Figs 3 and 4. Note that the Lineweaver–Burk plot in Fig. 4 is distinctly nonlinear.

The results are illustrated in Fig. 5, in which the white (open) symbols represent data points taken into the analysis and the black (filled) symbols represent data points that were purposely ignored. The truncated data set was subjected to model discrimination analysis using the statistical methods described above. Four standard inhibition mechanisms (competitive, uncompetitive, noncompetitive and mixed-type) were considered as alternatives. Two different statistical methods of model discrimination – Burnham & Anderson's [12] AIC-based approach, and Fisher's  $F$ -statistic for nested models [13] – both identified the competitive inhibition mechanism as the most preferred kinetic model.

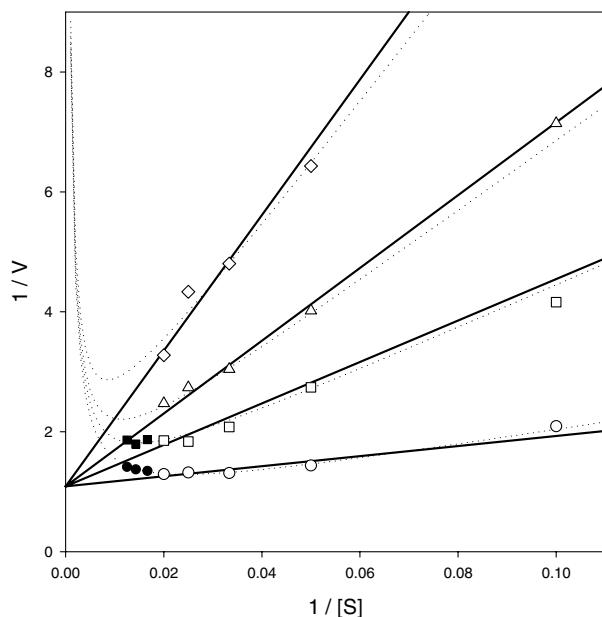
The corresponding double-reciprocal plots, used by Fridman *et al.* [3] for model identification, are shown



**Fig. 4.** Double-reciprocal Lineweaver–Burk plot corresponding to Fig. 3.



**Fig. 5.** Inhibition of the lethal factor (LF) protease by neomycin B: best least-squares fit of a truncated data set to the competitive model. The same experimental data were analyzed as those shown in Fig. 3. However, only the data points represented by the white (open) symbols were subjected to model discrimination analysis. The most plausible theoretical model is the competitive mechanism shown in Scheme 2, in agreement with previously published results for neomycin B [3]. Note that the ignored data points, represented by the black (closed) symbols, strongly indicate the involvement of substrate inhibition.



**Fig. 6.** Double-reciprocal Lineweaver–Burk plot corresponding to Fig. 5. Data points represented by the black (filled) symbols were ignored. For a detailed explanation, see the text.

in Fig. 6. We can see that if only the low substrate concentrations were taken into account, the inhibition mechanism would appear to be competitive, as shown by the double reciprocal plots intersecting on the vertical axis. We can also see in the double-reciprocal plots that the data points which deviate from the best-fit model (filled symbols in Fig. 6) do so much less visibly than in the direct plot in Fig. 5.

With regard to the molecular mechanism of lethal factor inhibition by aminoglycosides, we suggest that the discrepancy between the published mechanism for neomycin B (competitive) [3] and our results (mixed-type noncompetitive) can be explained in one of several ways. First, it is possible that neomycin B truly shows two different kinetic mechanisms of inhibition, depending on the nature of the substrate. For example, neomycin B could be noncompetitive with respect to the peptide substrate we used and competitive with respect to other substrates [3]. Second, it is possible that the previously reported kinetic mechanism is in error, because a limited range of substrate concentrations was used. Another source of erroneous model identification could be an improper analytical procedure employed for model identification (visual examination of double-reciprocal plots [3], as opposed to rigorous nonlinear regression in our study). In either case, our results and conclusions should be of interest to all researchers studying the lethal factor protease, or other enzymes

displaying substrate inhibition, with the aim of determining molecular mechanisms from kinetic data.

Yet another reason for the previous conclusions regarding the mechanism could be the nonlinearity of the reaction progress curves observed in lethal factor protease assays (data not shown). We found that it is essential to perform nonlinear fit of the reaction progress curves, rather than relying on routinely used linear fit of an arbitrarily chosen initial portion of each kinetic trace. Applying linear regression of the reaction progress could introduce a systematic error into the initial rates, which ultimately could result in the wrong molecular model being selected. This issue is discussed in detail by Cornish–Bowden ([14], pp. 40–42).

We suggest that there is a significant relationship between substrate inhibition observed for the synthetic peptide substrate used, and mixed-type noncompetitive inhibition observed for both inhibitors reported in this study. In particular, we note that the ratio of the substrate kinetic constants  $K_m : K_s$  is  $\approx 1 : 10$  ( $K_m = 8.6 \mu\text{M}$ ,  $K_s = 85 \mu\text{M}$ , see Fig. 1). Similar results regarding substrate inhibition in lethal factor kinetics were previously reported by Tonello *et al.* [16]. This suggests that at least some polycationic peptide substrates are binding to the lethal factor protease either at two different binding sites, or at the same binding site but in two different structural modes.

Similarly, the last column in Table 2 shows that the ratio of the two inhibition constants for both inhibitors varies between 1 : 5 and 1 : 11. This again suggests that the inhibitors bind to the enzyme either at two distinct binding sites, or at the same site but in two different orientations. For neomycin B, the principal binding site (or orientation) is formally characterized by the free energy of binding  $\Delta G_1 = -RT \ln K_i = -9.0 \text{ kcal}\cdot\text{mol}^{-1}$ , whereas the secondary binding mode is characterised by the free energy of binding  $\Delta G_2 = -RT \ln K_{is} = -7.5 \text{ kcal}\cdot\text{mol}^{-1}$ . Thus, the difference in binding energies ( $\Delta G_1 - \Delta G_2$ ) is  $\approx 1.5 \text{ kcal}\cdot\text{mol}^{-1}$ . In the case of neamine, we obtain  $\Delta G_1 = -6.7 \text{ kcal}\cdot\text{mol}^{-1}$  and  $\Delta G_2 = -5.8 \text{ kcal}\cdot\text{mol}^{-1}$ , less than a  $1.0 \text{ kcal}\cdot\text{mol}^{-1}$  difference. It is possible that these two distinct binding sites (or orientations) for the attachment of the inhibitor are somehow related to the two modes of substrate binding, which are manifested in substrate inhibition.

The synthetic substrate, a nonapeptide with an N-terminal *ortho*-aminobenzoyl and a C-terminal dinitrophenyl, contains three positively charged residues (lysine or arginine), similarly to the polycationic inhibitors. It is likely that the presence of multiple positive charges in both the substrate molecule and all the inhibitors are responsible for the similarity in their kinetic behavior.

At the molecular level, the inhibition pattern seen with the synthetic peptide substrate, in conjunction with the mixed-type noncompetitive inhibition pattern seen with neomycin B and other inhibitors, suggest either the presence of two distinct binding sites or the involvement of two alternate binding orientations at the same site. To help decide between these two possibilities, we employed a technique used previously to assess the effective electrical charge in the active site of acetylcholine esterase [8] and porcine pepsin [7]. Nolte *et al.* [8] studied ionic-strength effects on the inhibition of acetylcholine esterase by N-methylacridinium (electrical charge  $Z_L = +1$ ), and found that at the point of initial attachment, the enzyme and inhibitor molecules are separated by a  $d$  of  $\approx 14 \text{ \AA}$ . From the same data, these authors [8] concluded that the effective electrical charge on the active site is  $Z_E = -10$ . We previously used the same technique to study the inhibition of porcine pepsin by polycationic pseudo-peptide inhibitors [7] and found similar results ( $d = 26 \text{ \AA}$ ,  $Z_L \times Z_E = -19$ ). These data indicate that, for both enzymes, the attachment of cationic inhibitors to the negatively charged active site is governed by long-range, nonspecific electrostatic interaction.

In contrast, in the case of the lethal factor protease, our results reported here show that the binding of neomycin B and other cationic inhibitors is probably governed by short-range, specific electrostatic charges. This is seen in Fig. 2, where the plot of  $\sqrt{I.S.}$  against  $\ln(K_i^{(app)})$  for neomycin shows no curvature at all. Instead, the data points clearly fall on a straight line, suggesting that the  $d$ -value in Eqn (6) is zero. This, in turn, suggests the involvement of short-range, specific electrostatic binding. The relatively gentle slope of this plot means that only a single ionic-pair ( $Z_E Z_L \sim 1$ ) is probably involved in the enzyme–inhibitor interaction.

## Summary and conclusions

Based on the results of our model discrimination studies, and on the ionic strength effects on the apparent inhibition constants, we can conclude the following about the molecular mechanism by which the lethal factor protease from *B. anthracis* is inhibited by aminoglycosides:

- polycationic inhibitors, such as neomycin B, interact with the enzyme predominantly as a result of electrostatic (as opposed to hydrophobic or van der Waals) attractive interactions;
- these electrostatic interactions are probably specific and short range, rather than nonspecific;

- only a single ionic pair ( $Z_L = +1$  on the inhibitor,  $Z_E = -1$  on the enzyme) seems kinetically competent in inhibitor binding;
- the inhibitors probably bind to the specific site on the enzyme in two different orientations;
- the difference between the free energies of binding in the primary (strong, ‘competitive’) orientation and the secondary (weak, ‘uncompetitive’) orientation is  $\approx 1 \text{ kcal/mol}^{-1}$  for both inhibitors;
- the multiple modes of inhibitor binding correlate with the substrate inhibition seen with the polycationic substrate;
- ignoring the nonlinearity in the reaction progress curves from lethal factor assays systematically distorts the calculated initial reaction rates, which could lead to errors in the identification of the mechanism; and
- if an appropriate range of substrate concentrations is not used in kinetic experiments, it is possible to miss substrate inhibition, which causes the kinetic mechanism of inhibition to appear competitive, whereas including high substrate concentrations reveals the mixed-type noncompetitive mechanism.

## Experimental procedures

### Materials

Aminoglycosides were purchased from Sigma-Aldrich Corp. (St Louis, MO, USA) and from ICN (Irvine, CA, USA). The lethal factor protease and its fluorescence resonance energy transfer (FRET) peptide substrate, MAPKKide (*ortho*-aminobenzoyl/dinitrophenyl), were purchased from List Biological Laboratories (Campbell, CA, USA). Ninety-six-well half area plates for microplate assays were purchased from Fisher Scientific (Houston, TX, USA).

### Protease assays

The lethal factor protease was assayed according to the FRET method, first described for lethal factor protease by Cummings *et al.* [16]. Lethal factor protease (10  $\mu\text{L}$ , final concentration 10–20 nM, determined by active-site titration [17]) and inhibitor (5  $\mu\text{L}$ ) were briefly incubated at room temperature in the assay buffer (25  $\mu\text{L}$ , 20 mM Hepes, pH 7.4). The reaction was started by the addition of the fluorogenic peptide substrate (10  $\mu\text{L}$ , final concentration 12.5  $\mu\text{M}$ ). Fluorescence signal (excitation wavelength 320 nm, emission wavelength 420 nm) was monitored for 6–15 min at room temperature on the SpectraMax Gemini fluorescence plate reader (Molecular Devices, Sunnyvale, CA, USA). Raw data were exported from the SOFTMAX PRO software (Molecular Devices) and analyzed by using the software BATCHKI (BioKin Ltd, Pullman, WA, USA).

## Determination of apparent inhibition constants

The initial reaction rates ( $v_0$ ) were fit to the modified Morrison Eqn (1), according to the method described previously [19]. When appropriate, the  $[E]$  value was determined simultaneously with the determination of  $K_i^{(app)}$ ; the details of this simultaneous determination of  $[E]$  and  $K_i^{(app)}$  have been described previously [18].

## Confidence interval estimation

Nonsymmetrical 95% confidence intervals for the inhibition constants were computed by a systematic search of the multidimensional parameter space, according to a modification of the  $t$ -profile method of Bates & Watts ([20], pp. 205–214). In our modified computational procedure,  $t$ -profile plots were generated while holding all adjustable model parameters, except kinetic constants (e.g. adjustable concentrations and adjustable molar responses), at fixed values.

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## Disclaimer

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## References

- Duesbery NS, Webb CP, Leppla SH, Gordon VM, Klimpel KR, Copeland TD, Ahn NG, Oskarsson MK, Fukasawa K, Paull KD et al. (1998) Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. *Science* **280**, 734–737.
- Lee LV, Bower KE, Liang F-S, Shi J, Wu D, Scheck SJ, Vogt PK & Wong C-H (2004) Inhibition of the proteolytic activity of anthrax Lethal Factor by aminoglycosides. *J Am Chem Soc* **126**, 4774–4775.
- Fridman M, Belakhov V, Lee LV, Liang F-S, Wong C-H & Baasov T (2005) Dual effect of synthetic aminoglycosides: Antibacterial activity against *Bacillus anthracis* and inhibition of anthrax Lethal Factor. *Angew Chem Int Ed* **44**, 447–452.
- Tang C, Simo O, Nagata M, Jiao G-S, O'Malley S, Goldman M, Cregar L, Nguyen D & Hemscheidt T (2004) Guanidinylated neamine analogs inhibit LF with Kiapp of 1.5–25.0  $\mu$ M. Paper presented at the 228th ACS National Meeting. ACS, Philadelphia, PA.
- Turk BE, Wong TY, Schwarzenbacher R, Jarrell E, Leppla SH, Collier RJ, Liddington RC & Cantley LC (2004) The structural basis for substrate and inhibitor selectivity of the anthrax lethal factor. *Nat Struct Mol Biol* **11**, 60–66.
- Segel IH (1975) *Enzyme Kinetics*. Wiley, New York.
- Kuzmic P, Sun C-Q, Zhao Z-C & Rich DH (1991) Nonspecific electrostatic binding of substrates and inhibitors to porcine pepsin. *Adv Exp Med Biol* **306**, 75–86.
- Nolte HJ, Rosenbery TL & Neumann E (1980) Effective charge on acetylcholine esterase active site determined from the ionic strength dependence of association rate constants with cationic ligands. *Biochemistry* **19**, 3705–3711.
- Cha S (1975) Tight binding inhibitors. I. Kinetic behavior. *Biochem Pharmacol* **24**, 2177–2185.
- Kuzmic P (2006) A generalized matrix formalism for rapid equilibrium enzyme kinetics: Application to 17beta-HSD. *Mol Cell Endocrinol* **248**, 172–181.
- Kuzmic P (1996) Program DYNAFIT for the analysis of enzyme kinetic data: Application to HIV proteinase. *Anal Biochem* **237**, 260–273.
- Williams JW & Morrison JF (1979) The kinetics of reversible tight-binding inhibition. *Methods Enzymol* **63**, 437–467.
- Burnham KB & Anderson DR (2002) *Model Selection and Multimodel Inference: a Practical Information-Theoretic Approach*, 2nd edn. Springer-Verlag, New York.
- Mannervik B (1982) Regression analysis, experimental error, and statistical criteria in the design and analysis of experiments for discrimination between rival kinetic models. *Methods Enzymol* **87**, 370–390.
- Cornish-Bowden A (1979) *Fundamentals of Enzyme Kinetics*. Butterworths, London.
- Tonello F, Ascenzi P & Montecucco C (2003) The metalloproteolytic activity of the anthrax lethal factor is substrate-inhibited. *J Biol Chem* **278**, 40075–40078.
- Cummings RT, Salowe SP, Cunningham BR, Wiltsie J, Park YW, Sonatore LM, Wisniewski D, Douglas DM, Hermes JD & Scolnick EM (2002) A peptide-based fluorescence resonance energy transfer assay for *Bacillus anthracis* lethal factor protease. *Proc Natl Acad Sci USA* **99**, 6603–6606.
- Kuzmic P, Elrod KC, Cregar LM, Sideris S, Rai R & Janc JW (2000) High-throughput enzyme kinetics: Simultaneous determination of tight-binding inhibition constants and enzyme concentration. *Anal Biochem* **286**, 45–50.
- Kuzmic P, Sideris S, Cregar LM, Elrod KC, Rice KD & Janc JW (2000) High-throughput screening of enzyme inhibitors: Automatic determination of tight-binding inhibition constants. *Anal Biochem* **281**, 62–67.
- Bates DM, Watts DG (1988) *Nonlinear regression analysis and its applications*, Wiley, New York.