Polyamines Affect Differently the Antibiotic Potency

INSIGHT GAINED FROM KINETIC STUDIES OF THE BLASTICIDIN S AND SPIRAMYCIN INTERACTIONS WITH FUNCTIONAL RIBOSOMES*

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The effects of spermine on peptidyltransferase inhibition by an aminohexosylcytosine nucleoside, blasticidin S, and by a macrolide, spiramycin, were investigated in a model system derived from *Escherichia coli*, in which a peptide bond is formed between puromycin and AcPhe-tRNA bound at the P-site of poly(U)-programmed ribosomes. Kinetics revealed that blasticidin S, after a transient phase of interference with the A-site, is slowly accommodated near to the P-site so that peptide bond is still formed but with a lower catalytic rate constant. At high concentrations of blasticidin S (>10 × K<sub>i</sub>), a second drug molecule binds to a weaker binding site on ribosomes, and this may account for the onset of a subsequent mixed-noncompetitive inhibition phase. Spermine enhances the blasticidin S inhibitory effect by facilitating the drug accommodation to both sites. On the other hand, spiramycin (A) was found competing with puromycin for the A-site of AcPhe-tRNA-poly(U)-70 S ribosomal complex (C) via a two-step mechanism, according to which the fast formation of the encounter complex CA is followed by a slow isomerization to a tighter complex, termed C*A. In contrast to that observed with blasticidin S, spermine reduced spiramycin potency by decreasing the formation and stability of complex C*A. Polyamine effects on drug binding were more pronounced when a mixture of spermine and spermidine was used, instead of spermine alone. Our kinetic results correlate well with cross-linking and crystallographic data and suggest that polyamines bound at the vicinity of the antibiotic binding pockets modulate differently the interaction of these drugs with ribosomes.

Blasticidin S and spiramycin are representatives of two distinct antibiotic families, the aminohexosylcytosine nucleosides and the 16-membered lactone ring macrolides, respectively, both of which have been proposed to inhibit protein synthesis by binding to the large ribosomal subunit. Blasticidin S consists of a cytosine bonded to a pyranose ring, to which an amino acid-like substituent is attached (see Fig. 1). Therefore, it is not surprising that blasticidin S and puromycin have been considered as iso-structural, both with one another and with the 3'-end of aminocyl-tRNA (1, 2). Consistently, blasticidin S has been found to inhibit the binding of CACC(A) (Phe) to the A-site of ribosomes (3) and to compete with designated A-site inhibitors (4). In addition, the inhibition of peptidyl- or AcPhe<sup>3</sup> puromycin synthesis by this antibiotic shows a competitive phase in concert with noncompetitive or mixed-noncompetitive phases (5, 6), a fact supporting the notion that blasticidin S influences, at least transiently, the affinity of the A-site. Further support derives from chemical probing studies in *Escherichia coli* ribosomes (7), suggesting that blasticidin S protects A2439, one of the three nucleosides in domain V of 23 S RNA, which show altered chemical reactivity on removal of the aminoaicyl group from A-site-bound tRNAs (8). Moreover, A2439 is one of the preferable cross-linking sites for A-site-bound Phe-tRNA bearing a photoreactive group attached to its aminoaicyl terminus (9). In fact, the reactivity of A2439 is also affected by P-site-bound Phe-tRNA, and A2439 is susceptible to cross-linking by P-site-bound Phe-tRNA. The situation is further complicated by the finding of a recent crystallographic study in *Halocarcula marismortui*, revealing that blasticidin S binds at two, nonoverlapping sites, with different affinities (10). In the primary binding site (see Fig. 2A, site occupied by blasticidin I), the cytosine residue of blasticidin S is base-paired with G2251 (*E. coli* numbering). Further stabilization of the binding is conferred by hydrogen bonding of the N-methylguanidinium tail of blasticidin S with the backbone phosphates of A2439 and A2600. At this position, blasticidin S superimposes on C75 of a P-site substrate. Therefore, we should expect that blasticidin S might inhibit peptide bond formation by competing with the P-site substrate.

The spiramycin binding site has been localized by chemical probing (11) and crystallographic studies (12) within the exit-tunnel hydrophobic crevice. This is consistent with the long standing view that spiramycin acts during the early stages of protein synthesis by blocking the nascent polypeptide exit tunnel (13), a process that may also induce destabilization and premature dissociation of peptidyl-tRNAs from the ribosome (14). Recently, it was discovered that 16-membered macrolides also exhibit an inhibitory effect on 50 S ribosomal subunit assembly (15). Nevertheless, spiramycin can inhibit peptide bond formation in most model cell-free systems by direct block-

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The abbreviations used are: AcPhe, acetylphenylalanyl; Complex C, AcPhe-tRNA-poly(U)-70 S ribosome complex bearing AcPhe-tRNA at the P-site; PTase, peptidyltransferase; DMS, dimethyl sulfate; ABA, N<sup>-</sup>-azidobenzamidino; DR, double-reciprocal.
ing the PTase activity (11, 16, 17). This surprising effect seems to be due to the mycaminose/mycarose moiety of spiramycin, a disaccharide attached to the C5 position of the antibiotic (Fig. 1), which extending toward the catalytic center inserts into the A-site hydrophobic crevice (Fig. 2B). Chemical probing and cross-linking studies, reviewed by Spahn and Prescott (13), are in agreement with this consideration.

In the course of binding studies, most analyses have assumed that the interaction of ribosomes (R) with either blasticidin S or spiramycin (I) can be expressed by a fast equilibrium of the form, R + I ⇌ RI. However, this concession cannot justify the binding of blasticidin S at two, nonoverlapping sites, nor can it explain the strengthening of spiramycin potency after preincubation of ribosomes with the drug (16). Experiments from more than 30 years ago have also indicated that the ionic environment is an essential factor that influences both the PTase activity and the interaction of antibiotics with ribosomes (18, 19). Notably, spermine has been found to inhibit the interaction of ribosomes with erythromycin, an antibiotic sharing a set of common binding features with spiramycin (11). However, the effect of polyamines on the mode of action of blasticidin S and spiramycin has never been investigated.

In the present study, we utilize the puromycin reaction, a model reaction for peptide bond formation, under polyamine buffer conditions. By analyzing the peptide bond formation as a pseudo-first-order reaction, we reveal that blasticidin S, after a transient competitive phase, is slowly accommodated near to the P-site. By anchoring to this site, blasticidin S reduces the reactivity of P-site bound AcPhe-tRNA. At higher antibiotic concentrations, a second molecule of blasticidin S is attached to a secondary binding site, leading to mixed-noncompetitive inhibition. We also demonstrate that spiramycin interacts with ribosomes via a two-step mechanism, behaving as a slow binding inhibitor, namely the drug induces a slow conformational change in the ribosome upon binding, and this conformational change affects both the binding affinity and the pattern of inhibition. Polyamines attached in close proximity to the binding sites of blasticidin S and spiramycin affect the potency of these drugs differentially; they promote the inhibitory effect of blasticidin S but impair that of spiramycin. Our results are discussed in view of current crystallographic advances on the localization of these drugs on ribosomes.

MATERIALS AND METHODS

Reagents and Materials—Puromycin dihydrochloride (disodium salt), spermine tetrahydrochloride, spermidine trihydrochloride, DMS, DMS stop solution, heterogeneous tRNA from E. coli, spiramycin (mixture of spiramycins I, II, and III) were purchased from Sigma. Blasticidin S was kindly provided by Prof. H. Yonehara (University of Tokyo, Japan). L-[2,3,4,5,6-3H]Phenylalanine and [γ-32P]ATP were from Amersham Biosciences. Avian myeloblastosis virus reverse transcriptase and RNase H were obtained from Roche Diagnostics and Promega (Madison, WI), respectively. dNTPs and ddNTPs were from Roche Applied Science. Cellulose nitrate filters (type HA; 0.45-μm pore size) were from Millipore Corp. (Bedford, MA). ABA-spermine was synthesized and purified according to Clark et al. (20).

Biochemical Preparations—Salt-washed (0.5 M NH4Cl) 70 S ribosomes and partially purified translation factors were obtained from E. coli CAN20–12E cells as described elsewhere (6). Complex C, i.e. the Ac[3H]Phe-tRNA-poly(U)-70 S ribosome complex, was prepared in buffer A (100 mm Tris/HCl, pH 7.2, 6 mm magnesium acetate, 100 mm NH4Cl, and 6 mm 2-mercaptoethanol) containing partially purified translation factors and separated from excess Ac[3H]Phe-tRNA as reported previously (21). When desired, 100 μM spermine or 50 μM spermidine and 2 mM spermidine were also included in buffer A. In the purified product, ~20% of the used ribosomes were in the form of complex C. This fraction was almost fully reactive toward puromycin.

Photo-affinity Labeling, Chemical Modification, and Mapping of ABA-Spermine Cross-linking Sites in 23 S rRNA—Complex C was photolabeled with 100 μM ABA-spermine as described previously (22). After irradiation, the excess of ABA-spermine was inactivated by re-duction with 100 μM diethiothreitol and removed from complex C by gel filtration on Sephadex G-50 and microdialysis (22). Identification of spermine cross-linking sites was made by primer extension analysis.
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RESULTS

Inhibition of Peptide Bond Formation at Low Concentrations of Blasticidin S ([I] < 3 × Kᵢ)—The progress curve of AcPhe-puromycin synthesis carried out in buffer A, is expressed by a straight line at each concentration of puromycin. A representative plot obtained at 200 μM puromycin is given in Fig. 3A.
Fig. 3. Kinetic analysis of AcPhe-puromycin synthesis at low concentrations of blasticidin S ([I] < 3 × Kᵢ). A, first-order time plots. The reaction between complex C and 200 μM puromycin was carried out at 25 °C in buffer A, in the absence (○) or in the presence of blasticidin S at 0.2 μM (○), 0.4 μM (■), and 0.8 μM (□). B-D, DR plot, slope replott, and Hill plot analysis, respectively. The data presented in B and D were collected from the late phases (t > 1 min) of logarithmic time plots, such as those shown in panel A. The slope values indicated in panel C were estimated from the DR plots shown in panel B.

(upper line). However, when puromycin reaction is performed in the presence of blasticidin S, an early as well as a late phase can be clearly seen in the progress curves (Fig. 3A, three lower lines), a fact that reveals a delay in the onset of inhibition. In agreement with a previous study performed at 10 mM Mg²⁺ and 100 mM NH₄⁺ (6), analysis of the initial slopes by DR plotting (1/kₐobs, versus 1/[S]) and slope-replotting (slope of DR plots versus [I]), not shown, confirms that blasticidin S behaves as a competitive inhibitor over a narrow range of inhibitor concentrations ([I] < 3 × Kᵢ). The Kᵢ value found in the present study is approximately two-times higher than that reported in this earlier report (6). In contrast, our analysis of the late slopes revealed a partial-noncompetitive inhibition. As shown, with increasing concentrations of blasticidin S the apparent Kₚ value remains approximately constant (Fig. 3B), whereas the slopes of the DR plots are increased (Fig. 3C), tending to a limit. To evaluate the molecular order of blasticidin S participation in the puromycin reaction, a modified formula of Hill equation was used (25). A plot of this equation, obtained at 200 μM puromycin, is shown in Fig. 3D. The slope of this plot is equal to 0.75, a fact indicating that only one molecule of blasticidin S is involved in the mechanism of inhibition. Taken together, these results suggest that blasticidin S (I) reacts rapidly with complex C to form the encounter complex CI, which is then isomerized slowly to a tighter complex, termed C*I. In contrast to complex CI, complex C*I is capable to produce product, but with a lower catalytic rate constant than that of CS (control reaction). Therefore, a kinetic model that could adequately explain the above-mentioned results is that shown in Scheme I.

The Kᵢ*, kₖ*, and k₆/k₇ values estimated from 1/Δslope secondary replots (Fig. 4; see also Ref. 24) are presented in Table I. As described previously (26), the individual values of k₆ and k₇ can be calculated by nonlinear regression fitting of kinetic data to Equation 3,

\[ k' = k_7 + \frac{(k_6/[I]/K_I)}{1 + ([S]/K_S) + ([I]/K_I)} \]  
(Eq. 3)

where k' is the apparent equilibration rate constant for the attainment of equilibrium between complex C and the drug. The k' values were estimated from the intersection point of the vertical axis with the late linear part of the corresponding progress curve; at this point, the value of the vertical axis is related to the k' value by the relationship in Equation 4.

\[ \text{Intercept} = \frac{(k_{obs,0}) - (k_{obs})}{k'} \]  
(Eq. 4)

Equation 4 holds, provided that C*I is not reactive toward the substrate (26, 27). Therefore, to adopt precisely the slow onset inhibition theory in our analysis, the values of (k_{obs,0}) were first corrected by subtracting the contribution of k₆* step and then were fitted to Equation 4. The estimated k₆* and k₇ values are presented in Table I. Inhibition of Peptide Bond Formation at High Concentrations of Blasticidin S ([I] > 10 × Kᵢ)—Increase in the concentration of blasticidin S progressively alters the type of inhibition to mixed-noncompetitive (Fig. 5A). Analysis of this type of inhibition is described in detail by Segel (24). The change in the type of inhibition is also evident from the intercept replot, which is not linear (Fig. 5B). Linearity is established at concentrations of blasticidin S above 10 × Kᵢ. Because the linear part of the intercept replot does not extrapolate to the intercept of the control (1/k₉, according to Equation 2), but intersects the vertical axis at a point approximately equal to 1/k₉*, we surmise that the species, which interacts with S and I to give mixed-noncompetitive kinetics, is the tight complex C*I. This is also confirmed by the slope replot (Fig. 5C), whose intercept equals 15 μM/min. This value is about similar to that calculated for the K₉*/k₆* ratio. The [I] axis intercept of the intercept replot gives a value for the equilibrium constant αK₉* equal to 18 μM. This value is about 2.4 times higher than the K₉* value estimated from the [I] axis intercept of the slope replot (Fig. 5C). A model adequately explaining the above-mentioned kinetic results is presented in Scheme II.
Data were collected from the initial phases (time plots). The reaction was carried out at 25 °C in buffer A, in the absence (C) or in the presence of blastidin S at 6 mM (■), 2.5 mM (□), 4.0 mM (▲), 6 mM (○), 8 mM (△), and 12 mM (▲). B, intercept replot. The values of 1/b on were estimated from the intercepts of DR plots presented in panel A and similar plots. C, slope replot. The slope values were estimated from DR plots shown in panel A.

According to this model, blastidin S exhibits a transient phase of competitive inhibition followed by a slow isomerization of complex CI to C*I. The latter complex is tighter than CI and also capable to accommodate puromycin as well as a second molecule of the drug. Because the $K_{i*}$ value is about 75 times higher than the value of $K_i$ at $k_{on} + k_{off}$, which represents the overall inhibition constant of the first binding site, we conclude that the second binding site of blastidin S is much weaker than the first one.

**Polyamines Amplify the Inhibitory Effect of Blastidin S at 6 mM Mg$^{2+}$**—To evaluate the effect of spermine on the blastidin S potency, we re-examined the mechanism of inhibition, using complex C prepared and interacting with puromycin in buffer A containing 100 μM spermine. Addition of spermine at this concentration improves the activity of PTase (Table I) but does not change the type of inhibition by blastidin S. Nevertheless, the values of certain kinetic parameters differ from those observed in the absence of spermine (Table I). Namely, the $K_i$ value is decreased from 0.38 to 0.14 μM. In addition, the $k_{on}$ value is enhanced, whereas the $k_{off}$ value remains essentially unchanged. As a consequence, the overall inhibition constant of the primary binding site, $K_i [k_{on} k_{off} + k_{off}]$, becomes four times lower. Moreover, the $k_{on}$ and $K_{i*}$ values are reduced, whereas the $K_{i*}$ value is slightly increased. Similar changes in the values of the kinetic constant are recorded if spermine is covalently attached by photolabeling to complex C or if the polyamine buffer contains 50 μM spermine and 2 mM puromycin. In the latter case, the observed changes in the $K_i$ and $k_{on}$ values are more pronounced (Table I). However, again the type of inhibition is not altered.

**Inhibition of Peptide Bond Formation by Spiramycin**—In agreement with previous results obtained at 10 mM Mg$^{2+}$ (16), spiramycin inactivates complex C at 6 mM Mg$^{2+}$, almost irreversibly. Each time the plot reaches a plateau, which progressively bends down with increasing drug concentrations (plots not shown). Additional evidence in support of the mechanism of spiramycin interaction with complex C was sought by exposing complex C to various spiramycin concentrations for several time intervals and titrating the remaining catalytic activity by puromycin. Such inactivation plots are shown in Fig. 6A. All of them give a straight line for each concentration of spiramycin, intersecting the vertical axis at a common point. This inhibition pattern suggests that the inactivation of complex C by spiramycin follows first-order kinetics. From the slope of each straight line, we can obtain a pseudo-first-order rate constant of inactivation ($k_{in}$), whose reciprocal, if plotted against 1/spiramycin, gives a straight line intercepting at a point above zero (Fig. 6B). This finding reveals that the inactivation of complex C by spiramycin (A) proceeds via a two-step mechanism (Scheme III).

**Scheme III**

Provided that the value of $k_{in}$ is very low, the relationship,

$$k_{in} = \frac{k_{off}[A]}{K_i + [A]}$$

(Eq. 5)
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Polyamines Reduce the Binding of Spiramycin to Complex C—Early reports (18, 19) provide evidence that the interaction between macrolide antibiotics and ribosomes is influenced by the ionic environment. Specifically, Teraoka and Tanaka (19) demonstrated that spermine inhibits the binding of erythromycin to ribosomes from E. coli. They also observed that this inhibitory effect is diminished at high concentrations of Mg\(^{2+}\) and monovalent cations. It should be mentioned that erythromycin and spiramycin compete for overlapping binding sites on the large ribosomal subunit (11). These observations prompted us to re-examine the interaction of spiramycin with complex C, in the presence of spermine. By repeating the experiments at 100 \(\mu M\) spermine (in buffer A), we found that the \(K_A\) value is reduced by 50\%, a fact indicating a beneficial effect of spermine on the formation of the encounter complex CA. In contrast, the effect of spermine on the subsequent isomerization step is detrimental; the \(k_{on}\) value is decreased by 75\%, whereas the \(k_{off}\) value becomes 2-fold higher (Table I). As a consequence, the ratio \(k_{on}/k_{off}\) is decreased from 333.3 to 46.2. Data obtained with complex C photolabeled by 100 \(\mu M\) ABA-spermine are in agreement with the above-mentioned results (Table I). By using 50 \(\mu M\) spermine and 2 \(mM\) spermidine, the \(k_{on}\) value is further decreased (Table I).

ABA-Spermine Cross-linking with 23 S rRNA Interferes with Blasticidin S and Spiramycin Binding—ABA-spermine is a photoreactive analogue of spermine, which has an arylnitrazo group attached to one of the terminal amino groups of the molecule (29). Previous studies of our group have demonstrated that ABA-spermine retains almost all biochemical properties of the parent compound (29, 30). On the other hand, photolabeled complex C with ABA-spermine exhibits similar kinetic properties in peptide-bond formation to those obtained with untreated complex C reacting with spermine free in solution (30). Therefore, it is reasonable to believe that ABA-spermine binds specifically to the ribosome.

ABA-spermine attached to a nucleoside of 23 S rRNA acts as a barrier for reverse transcriptase (22). Thus, the site of cross-linking can be localized exactly by primer-extension analysis.

In the present study, such experiments have been focused on 23 S rRNA regions to which blasticidin S and spiramycin bind. Representative autoradiograms are shown in Fig. 7 (A and B). The nucleosides modified with ABA-spermine are summarized in Fig. 7C. It is obvious that some of them coincide or are in close proximity to nucleosides implicated in blasticidin S and spiramycin binding. It is also evident that spermine cross-linking alters the reactivity of certain nucleosides in the V-loop toward DMS. Except for 12 positions found previously (26), additional sites are identified by the present study, located in helices H74 and H80. Namely, the reactivities of A2058 and A2024 increase upon ABA-spermine cross-linking, whereas the reactivities of A2058, A2059, and A2062 decrease. Taking into account that these sites are not directly related to ABA-spermine photo-incorporation, we suppose that the observed alterations in reactivity are due to 23 S rRNA conformational changes.

Discussion

Despite the fact that much has been accomplished in revealing the polynucleotide action on regulation of the translation process at several levels (31–34), there is still relatively little information available concerning the influence of polynucleotides on the interaction of ribosomes with antibiotics. In a recent report, we presented evidence that spermine bound to ribosomes promotes the binding of chloramphenicol (26), a drug primarily interfering with the A-site (35). In the current report, we extend this study by examining the effect of polynucleotides on the binding of two other antibiotics, blasticidin S and spiramycin, which bind the large ribosomal subunit at the P-site and at the entrance to the exit tunnel, respectively. At the same time, we seize the chance to revisit the mechanism of action of these two antibiotics on peptide-bond formation, in light of the high resolution crystal structures for their binding sites on the ribosome (10, 12).

According to the results of this study, blasticidin S behaves as a slow binding inhibitor. Such inhibitors have been characterized and named by Morrison and Walsch (27) to convey the idea that binding, which is the establishment of the equilibria between enzyme, inhibitor, and enzyme-inhibitor complexes, occurs slowly, in the time scale of seconds to minutes. To explain this behavior, Morrison and Walsch assumed that the inhibitor induces a slow conformational change in the enzyme, and this conformational change affects both the binding affinity and the inhibition pattern. In the case of blasticidin S, two molecules of the drug are involved in the mechanism of inhibition. The first molecule interferes with the binding of puromycin to the A-site (competitive kinetics) transiently. In fact,
interactions of blasticidin S with the A-site were not identified in the one published crystallographic study (10), probably because the competitive phase is transient. Soon after this initial competitive interaction, the antibiotic is slowly accommodated in its final position (C*I complex), so that puromycin is accepted and peptide bonds are still formed but with a lower catalytic rate constant (partial-noncompetitive kinetics). The type of inhibition and the tightness of complex C*I prompt us to suppose that the binding site of blasticidin S in C*I complex coincides with that of blasticidin I (Fig. 2A), identified by crystallographic studies (10). On the basis of the above observations we assume that blasticidin S (blasticidin I in Fig. 2A) by interacting with G2251 of the P-loop, orientates the aminoacyl group of P-site bound AcPhe-tRNA toward a less active position of the catalytic cavity. The accommodation of the drug in this binding site is accomplished via a two-step mechanism. This is supported by the finding that biphasic logarithmic time plots are obtained and that \( k_{\text{obs}} \) is reduced by increasing drug concentrations (Fig. 3A). The apparent association rate constant of binding, \( k_a = \frac{k_f + k_d}{K_i} \), equals 1.36 \( \times \) \( 10^6 \) M\(^{-1}\) s\(^{-1}\), a value lower than the upper limit of 10\(^6\) M\(^{-1}\) s\(^{-1}\) set for the classification of a drug as a slow binding inhibitor (27). In addition, the \( k_f \) value is less than that measured for the forward rate constant, \( k_c \). Consequently, our results suggest that a fast association-dissociation equilibrium step (competitive phase) is followed by a slower rearrangement step, which falls within the minute time scale.

At high concentrations of blasticidin S (\(|I| > 10 \times K_i \)), the C*I complex can accept a second molecule of inhibitor, but then peptide bonds are not formed (complete, mixed-noncompetitive inhibition). The binding of the second molecule of blasticidin S is characterized by a higher inhibition constant value (\( K_i^* = 7.4 \mu M \)), which means that the second binding site appears to have much lower affinity for blasticidin S. Probably, this site coincides with that occupied by blasticidin II in Fig. 2A. Simultaneous occupation of both sites by the drug would prevent the interactions of P-site substrate with both G2251 and G2252 and thereby orientates the CCA sequence of P-site bound AcPhe-tRNA outside the PTase center (complete inhibition).

The affinity of complex C for blasticidin S is enhanced by addition of 100 \( \mu M \) spermine or by prelabeling complex C with 100 \( \mu M \) ABA-spermine (Table I). The use of an optimized polyamine buffer (32) containing 50 \( \mu M \) spermine and 2 mM spermidine improves even more the affinity than spermine alone (Table I). The beneficial effect of polyamines is more pronounced during the binding of the first molecule of inhibitor. Namely, the value of the overall inhibition constant, \( K_i[k_f + k_d + k_j] \), observed in the presence of polyamines, is \( 4 \)-fold lower than that found in conventional buffer. Instead, the \( K_i^* \) value that corresponds to the binding of the second molecule of blasticidin S (blasticidin II in Fig. 2A) is reduced by only 53%. Consistently, ABA-spermine cross-links were found at positions 2249, 2439, and 2601. Nucleotide U2249 is in close proximity to G2251 and G2252. These are two critical nucleosides of the P-loop of 23 S rRNA that fix the CCA-3'-end of P-site bound substrate in the PTase center by forming Watson-Crick base pairs with C75 and C74 of tRNA, respectively (36, 37). The importance of these nucleosides in blasticidin S binding is beyond doubt (Fig. 2A (10)). It is tempting to suppose that spermine, attached to U2249 and extending as far as the G2251 and G2252 positions, may influence the interaction of both drug molecules, blasticidin I and blasticidin II, with ribosomes.
either directly or by altering the local conformation of ribosomes. Evidence supporting the latter hypothesis is provided by chemical probing with DMS indicating that spiramycin cross-linking increases the reactivity of the adjacent A2247 nucleoside. With respect to A2439, various experimental approaches, including footprinting (7), mutational (38), and x-ray crystallographic analyses (10), have demonstrated that this bulged nucleoside as well as the adjacent U2438 are implicated in the binding of aminocyclaminonucleoside antibiotics. Both nucleosides are very susceptible to metal ion-catalyzed hydrolysis (39) and probably constitute a general cation binding site. Especially, Hansen et al. (10) have indicated that the N-methylguanidinium tail of blasticidin S bound at the primary site (blasticidin S I in Fig. 2A) interacts hydrophobically with the base of A2439 and forms hydrogen bonds with the backbone phosphates of A2439 and A2600. The latter observation also emphasizes the role of ABA-spermine cross-linking to C2601. In combination, these findings reveal the interdependence of blasticidin S binding and polyamine environment.

In accordance with previous studies (11, 16), spiramycin inhibits the puromycin reaction, producing nonlinear progress curves whose initial slope and plateau level vary as a function of the inhibitor concentration. These results are compatible with spiramycin behaving as a slow binding, near irreversible inhibitor. In fact, a very slow regenerations of activity from the inhibitor. In fact, a very slow regeneration of activity from the ionic environment should be taken into account. The Ribosome: Structure, Function, and Evolution (Hill, W., Dahlberg, A., Garrett, R., Moore, P., Schlessinger, D., and Warner, J. eda) pp. 592–510, AMS Press, Washington, D.C.

3. Pestka, S. (1974) Methods Enzymol. 30, 261–282

4. Pestka, S. (1974) Antimicrob. Agents Chemother. 5, 255–267

5. Pestka, S. (1975) J. Biol. Chem. 247, 4669–4678

6. Kalpakis, D. L., Theocharis, A., D., and Coutsogeorgopoulos, C. (1986) Eur. J. Biochem. 154, 267–271

7. Rodriguez-Fonseca, C., Amils, R., and Branca, C. (1993) Nucleic Acids Res. 21, 887–966

8. Hansen, J. L., Moore, P., and Steitz, T. A. (2003) J. Mol. Biol. 330, 1061–1075

9. Poulsen, S. M., Kofoed, C., and Vester, B. (2000) J. Mol. Biol. 304, 471–481

10. Hansen, J. L., Ippolito, J. A., Ban, N., Nissen, P., Moore, P. B., and Steitz, T. A. (2002) Mol. Cell 10, 117–128

11. Hanshaw, D., and Noller, H. F. (1998) Cell 97, 585–597

12. Mitchell, P., Stade, K., Osvald, M., and Bronmacome, R. (1993) Eur. J. Biochem. 21, 811–818

13. Champsy, W. (2001) Curr. Drug Targets Infect. Disord. 1, 19–36

14. Dinos, G., Syntetos, D., and Coutsogeorgopoulos, C. (1993) Biochemistry 32, 10638–10647

15. Kirillov, S. V., Porse, B. T., and Garrett, R. A. (1999) RNA (N. Y. 5) 5, 1003–1013

16. Vogel, Z., Vogel, T., Zmar, A., and Elson, D. (1971) J. Biol. Chem. 246, 339–346

17. Teraoka, H., and Tanaka, K. (1973) Eur. J. Biochem. 38, 578–583

18. Clark, E., Swank, R. A., Moore, P. B., and Hill, W., Dahlberg, A., Garrett, R., Moore, P., Schlessinger, D., and Warner, J. (eds) (1993) Nucleic Acids Res. 21, 5074–5083

19. Blair, J. T., and Walsh, C. T. (1988) Adv. Enzymol. Relat. Areas Mol. Biol. 61, 201–301

20. Erion, M. D., and Walsh, C. T. (1987) Biochemistry 26, 3417–3425

21. Trieman, K. L. (1993) Methods Enzymol. 164, 650–658

22. Cohen, S. S. (1998) A Guide to Polyamines, pp. 495–506, Oxford University Press, New York, NY

23. Agarwal, R. K., Pentz, P., Grasu, R. A., Burkhardt, N., Nierhaus, K. H., and Frank, J. (1999) J. Biol. Chem. 274, 8723–8729

24. Schlunzen, P., Zavatsch, F., Zahn, J., Zabre, A., Tocci, J., Albrecht, R., Yonath, A., and Frank, J. (1991) Nature 413, 814–821

25. Samaha, R. R., Green, R., and Noller, H. F. (1995) Nature 377, 309–314

26. Nissen, P., Hansen, J., Ban, N., Moore, P., and Steitz, T. (2001) Science 289, 920–930

27. Triman, K. L. (1993) Adv. Genet. 31, 157–195

28. Polacek, N., and Bartetzko, A. (2000) Antimicrob. Agents Chemother. 45, 1–12

29. Depadu, F., and Courval, P. (2001) Antimicrob. Agents Chemother. 45, 319–323