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Kinetics of Inhibition of Rabbit Reticulocyte Peptidyltransferase by Anisomycin and Sparsomycin

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ABSTRACT

A detailed kinetic study was carried out on the inhibitory mechanisms of two eukaryotic peptidyltransferase drugs (I), anisomycin and sparsomycin. In an *in vitro* system from rabbit reticulocytes, AcPhe-puromycin is produced in a pseudo-first-order reaction from the preformed AcPhe-tRNA/poly(U)/80S ribosome complex (complex C) and excess puromycin (S). This reaction is inhibited by anisomycin and sparsomycin through different mechanisms. Anisomycin acts as a mixed noncompetitive inhibitor. The product, AcPhe-puromycin, is derived only from C according to the puromycin reaction. On the other hand, sparsomycin reacts with complex C in a two-step reaction,

$$C + I \underset{}{\overset{K_i}{\rightleftharpoons}} CI \overset{k_6}{\underset{k_7}{\rightleftharpoons}} C^*I$$

An initial rapid binding of the drug produces the encounter complex CI. During this step and before conversion of CI to C*I, sparsomycin behaves as a competitive inhibitor. The rapidly produced CI is isomerized slowly to a conformationally altered species C*I in which I is bound more tightly. The rate constants of this step are $k_6 = 2.1\ min^{-1}$ and $k_7 = 0.095\ min^{-1}$. Moreover, the low value of the association rate constant k_7/K_i' ($2 \times 10^5\ M^{-1}\ sec^{-1}$), provides insight into the rates of possible conformational changes occurring during protein synthesis and supports the proposal that sparsomycin is the first example of a slow-binding inhibitor of eukaryotic peptidyltransferase. When complex C is preincubated with concentrations of sparsomycin of $>8\ K_i$ and then reacts with a mixture of puromycin and sparsomycin, the inhibition becomes linear mixed noncompetitive and involves C*I instead of CI. During this phase, AcPhe-puromycin is produced from a new, modified ribosomal complex with a lower catalytic rate constant. Thus, sparsomycin also acts as a modifier of eukaryotic peptidyltransferase activity.

Antibiotics have been used as tools to probe ribosomal structure and function. These studies have been conducted mainly in prokaryotes, either as binding studies on free ribosomes or as inhibition studies on polypeptide-synthesizing systems (Vazquez, 1979). More recently, studies to locate the catalytic center of ribosomal peptidyltransferase have been aided by RNA-footprinting and by cross-linking studies of antibiotic/ribosome complexes (Noller, 1991; Rodriguez-Fonseca *et al*., 1995). These studies often implicated nucleotides that lie within highly conserved sequences in 23S rRNA. In fact, all of the footprints characterized for several inhibitors of peptidyltransferase are concentrated in, and around, the central loop of domain V of 23S rRNA (Moazed and Noller, 1987; Egebjerg and Garrett, 1991; Douthwaite, 1992; Vannuffel *et al*., 1992; Garrett and Rodriguez-Fonseca, 1995).

The antibiotic anisomycin initially was described as a classic protein synthesis inhibitor on eukaryotic (80S) ribosomes, whereas it is inactive on prokaryotic (eubacterial) 70S ribosomes (Pestka *et al*., 1972; Barbacid and Vazquez, 1974a). The precise manner in which it exerts its inhibition remains largely unknown. There is general agreement that anisomycin acts on the large ribosomal subunit and inhibits peptide bond formation (Gale *et al*., 1981). Subsequently, it was found to inhibit growth and the *in vitro* protein synthesis in several archaebacteria, although they possess 70S ribosomes (Elhardt and Böck, 1982). These facts led to the contention that the anisomycin interaction site on the 70S ribosomes of archaebacteria may have structural features typical of eukaryotic 80S ribosomes (Hummel and Böck, 1985). It was found that anisomycin is one of the antibiotics that produce clear footprints on 23S-like rRNA in archaebacteria, thus implying that rRNA plays an important role in the interaction of this drug with the ribosome (Hummel and Böck, 1987).

The antibiotic sparsomycin, on the other hand, is a universal and powerful inhibitor of peptide bond formation and one of the most extensively studied inhibitors of protein synthesis. This is consistent with it acting at a highly conserved functional site. It seems that sparsomycin interferes initially with the ribosomal A site competing with the antibiotic puromycin and, thus, with aminoacyl-tRNA (Ottenheijm *et al*., 1986; Theocharis and Coutsogeorgopoulos, 1992). The exact nature of the sparsomycin binding site remains unknown. Studies with sparsomycin analogues showed that hydrophobic interactions play a crucial role in drug binding, suggesting that ribosomal proteins may be also involved in formation

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of the drug-binding site (Lazaro *et al.*, 1991a). In contrast to many other antibiotics, including anisomycin, sparsomycin does not produce footprints on RNA (Moazed and Noller, 1991). Recently, mutation C2518U (Tan *et al.*, 1996) and the lack of a modification in U2603 of halobacterial 23S rRNA (Lazaro *et al.*, 1996) were found to confer resistance to sparsomycin. These two nucleotides belong to the central loop of domain V of halobacterial 23S rRNA. Therefore, rRNA plays an important role in binding, even of the antibiotics that do not produce rRNA footprints.

During the past decade, we have carried out studies on the inhibition by several antibiotics of peptide bond formation in *Escherichia coli*. For this purpose, we used a ribosomal ternary complex from *E. coli* (complex C) in which the donor AcPhe-tRNA is reactive toward puromycin and forms peptide bonds in AcPhe-puromycin. The reaction of complex C with excess puromycin, the so-called puromycin reaction, can be conveniently analyzed as a pseudo-first-order reaction (Synetos and Coutsogeorgopoulos, 1987). Subsequently, the inhibition of this reaction by several antibiotics was examined. These studies provided evidence that most of these antibiotics, including sparsomycin, do not behave as classic competitive inhibitors of the prokaryotic peptidyltransferase but instead belong to the class of slow-binding inhibitors (Morrison and Walsh, 1988).

We recently reported the development of a eukaryotic cell-free system (Ioannou *et al.*, 1997), similar to that from *E. coli*, that is amenable to a kinetic analysis similar to the one that has been successfully applied to the *E. coli* system (Synetos and Coutsogeorgopoulos, 1987). Using this system, we carried out for the first time a detailed analysis of the mechanism of inhibition of the puromycin reaction by a eukaryotic protein synthesis inhibitor such as anisomycin. Moreover, and to conclude the kinetic study of sparsomycin as protein synthesis inhibitor, it was examined whether it behaves in eukaryotes as a classic (fast) or slow-binding inhibitor. Finally, we compared the mechanism of action of anisomycin and sparsomycin. These two drugs are active in different cell types; differences in their mechanism of action on rabbit reticulocyte ribosomes might indicate distinct sites of action for the two antibiotics.

Experimental Procedures

Materials. L-*phenyl*[2,3-^{3}H]Alanine was purchased from Amersham (Buckinghamshire, UK). Poly(U), GTP (disodium salt), ATP (disodium salt), phenylalanine, puromycin dihydrochloride, and heterogeneous tRNA from *E. coli* strain W were from Sigma Chemical (St. Louis, MO). Zwittergent 3–12 detergent (*N*-dodecyl-*N*,*N*-dimethyl-3-ammonium-1-propanesulfonate) was obtained from Calbiochem (San Diego, CA). Cellulose nitrate filter disks (type HA, 24-mm diameter, 0.45-μm pore) were purchased from Millipore (Bedford, MA). Sparsomycin and anisomycin were a gift from Upjohn (Kalamazoo, MI).

Formation of complex C. Crude rabbit reticulocyte ribosomes, which were not washed with 0.5 M KCl, were isolated according to the method of Allen and Schweet (1962). Crude Ac-[^{3}H]Phe-tRNA charged with 14.9 pmol of [^{3}H]Phe (170,000 cpm total)/A_{260} unit was prepared as described previously (Coutsogeorgopoulos *et al.*, 1972). Complex C (i.e., the Ac-[^{3}H]Phe-tRNA/poly(U)/80S ribosome complex) was formed as described by Ioannou *et al.* (1997). Briefly, it was formed in a 200-μl binding mixture containing 50 mM Tris·HCl, pH 7.6, 10 mM $MgCl_2$, 100 mM KCl, 5 mM 2-mercaptoethanol, 0.24 mg/ml poly(U), 0.4 mM GTP, 1.4 A_{260} units of unwashed 80S ribosomes, and 23.8 pmol (11,400 cpm/pmol) of Ac-[^{3}H]Phe-tRNA. After incubation at 37° for 30 min, complex C was separated from excess donor Ac-[^{3}H]Phe-tRNA present in the binding mixture by dilution with ice-cold buffer A {50 mM Tris·HCl, pH 7.6, 10 mM $MgCl_2$, 100 mM KCl, 5 mM mercaptoethanol}, filtration through cellulose nitrate filter disks, and three washes with buffer A. This complex was reacted with a mixture of puromycin and anisomycin or sparsomycin. Alternatively, complex C was preincubated with the inhibitor and then reacted with a mixture of puromycin and inhibitor (see Puromycin reaction). When required, complex C was desorbed into a solution containing the detergent Zwittergent 3–12 (extract) and then reacted with puromycin (Ioannou *et al.*, 1997).

Formation of a mixture of complex C and the sparsomycin complex. For the regeneration of complex C experiments, sparsomycin at a final concentration of 1×10^{-6} M was added to the binding mixture that contained preformed complex C. The new binding mixture was incubated for an additional 10 min at 25° and then filtered and processed as described previously for complex C. In this way, a mixture was obtained of complex C and the sparsomycin complex C*I.

Puromycin reaction. In the absence of inhibitor (I), the reaction between the disk-adsorbed complex C and puromycin was carried out at 25° as reported elsewhere (Ioannou *et al.*, 1997). In the presence of anisomycin or sparsomycin, the puromycin reaction was carried out under two different conditions:

The first condition was without preincubation of the disk-adsorbed complex C with I. The puromycin reaction was started by adding a half-disk bearing complex C to buffer A (1.0 ml) containing puromycin and anisomycin or sparsomycin at the desired concentrations. The reaction was allowed to proceed at 25° for the time intervals indicated and was stopped by the addition of 1.0 ml of 1.0 N NaOH. Shaking continued at 25° for 30 min to ensure hydrolysis of the unreacted Ac-[^{3}H]Phe-tRNA to Ac-[^{3}H]Phe and tRNA. The percentage of the ribosome-bound Ac-[^{3}H]Phe-tRNA that reacted with puromycin was corrected with the extent factor α ($x' = x/\alpha$) and determined as described previously (Ioannou *et al.*, 1997).

The second condition was after preincubation of the disk-adsorbed complex C with I. In this procedure, the half-disk bearing complex C initially was preincubated at 25° for 10 min in a total volume of 0.9 ml of buffer A containing anisomycin or sparsomycin at the desired concentrations. Puromycin in a mixture with the inhibitor was added in a volume of 0.1 ml, so the final reaction mixture (1.0 ml) contained the desired concentration of puromycin and anisomycin or sparsomycin. Subsequent steps are identical to those described for the first condition.

First-order analysis of the puromycin reaction. In the absence of inhibitor, the reaction between the disk-adsorbed complex C and excess puromycin displays pseudo-first-order kinetics (Ioannou *et al.*, 1997). Briefly, at a fixed initial concentration of puromycin (S), the corrected value of $x/\alpha = x'$ was obtained for various time intervals (t) and fitted into the integrated law of a first-order reaction such as $k_{obs} \cdot t = \ln [100/(100 - x')]$, which represents a straight line. The slope of this straight line gives the value of k_{obs} at each concentration of puromycin (S). The relationship $k_{obs} = k_3 \cdot [S]/(K_s + [S])$ holds, and from the double-reciprocal plot, the values of k_3 and K_s can be obtained. In the presence of an inhibitor (I), the first-order rate constant (k) is given by the equation $\ln [100/(100 - x')] = k \cdot t$. In the presence of sparsomycin, the time plots may be biphasic depending on the concentrations of puromycin and sparsomycin. In such cases, the slope of the line going through the origin (initial slope of the time plot) is taken as the value of k (i.e., the value of k_{obs} in the presence of I). The relationship between k and S follows the equation $k = k_{max} \cdot [S]/(K_s' + [S])$, where K_s' is the apparent K_s value in the presence of (I) and depends on the drug concentration.

Determination of the apparent inactivation rate constant k_{obs}^I (inactivation plots). Buffer A (0.9 ml) containing sparsomycin at the desired concentration was added to each one of a series of small beakers and allowed to equilibrate at 25° for 5 min. One half of a

cellulose nitrate filter disk bearing complex C was added to each beaker and the sparsomycin reacted with complex C for various time intervals. After the desired reaction time had elapsed, 0.1 ml of 20×10^{-3} M puromycin (containing the appropriate amount of sparsomycin so the final concentration remains constant) was added. Puromycin was allowed to react (backtitration with puromycin) for 30 sec, and then 1.0 ml of 1 M NaOH was added. Shaking continued at 25° for 30 min to ensure hydrolysis of the unreacted Ac-[^{3}H]Phe-tRNA. The corrected percentage $x/\alpha = x'$ for each time of exposure (t) of complex C to sparsomycin was plotted against t at each concentration of inhibitor. From these plots, the values of x' at equilibrium (x'_{eq}) can be obtained. For each concentration of sparsomycin, an apparent k^I_{obs} value was determined from the slope of the plot of $\log(x' - x'_{eq})$ versus time (t), assuming that the reaction between complex C and sparsomycin proceeds toward equilibrium as a pseudo-first-order reaction.

Regeneration of complex C from the sparsomycin complex. Sparsomycin complex in a mixture with complex C was isolated on a cellulose nitrate filter disk, free of excess sparsomycin, as described. This mixture was exposed to buffer A for various time intervals at 25°. At the end of each exposure, the cellulose nitrate filter was removed from the buffer, and the regenerated complex C was titrated with 2×10^{-3} M puromycin for 2 min at 25°.

Results

Inhibition of the Puromycin Reaction by Anisomycin and Sparsomycin

The inhibition of peptide bond formation on rabbit reticulocyte ribosomes by the antibiotics anisomycin and sparsomycin was studied in an *in vitro* system in which eukaryotic complex C was isolated on cellulose nitrate filter disks free of excess unbound AcPhe-tRNA. The complex then reacted with excess puromycin (S) according to the ribosome-catalyzed reaction shown in eq. 1:

$$C + S \underset{}{\overset{K_s}{\rightleftharpoons}} CS \xrightarrow{k_3} C' + P \qquad (1)$$

in which $K_s = 6.7 \times 10^{-4}$ M and $k_3 = 1.67\ min^{-1}$ (Ioannou *et al.*, 1997).

The progress of this reaction is monitored by the determination of AcPhe-puromycin (P), which carries a peptide bond. Because C′ cannot revert back to C, the puromycin reaction can be analyzed as a pseudo-first-order reaction, giving logarithmic time plots that are linear.

Fig. 1A shows the time course of the reaction between complex C from rabbit reticulocytes and puromycin in the absence or presence of increasing concentrations of anisomycin. In both cases, the time plots are straight lines until all of complex C has been converted to product for all antibiotic concentrations tested. This linearity provides evidence that the puromycin reaction remains first-order in the presence of anisomycin and that the same rate law applies throughout the reaction. The degree of inhibition depends only on the concentration of the inhibitor because incubation of complex C with anisomycin before the addition of puromycin did not change the degree or type of inhibition.

Fig. 1B depicts the time course of the reaction between complex C and puromycin in the absence or presence of increasing concentrations of sparsomycin. In the absence of the drug, a straight line is obtained until all of the AcPhe-tRNA in complex C has been converted to AcPhe-puromycin. In the presence of sparsomycin, however, the reaction becomes slower, and biphasic time plots are obtained showing that the degree of inhibition changes with time (time-dependent inhibition). The deviation from linearity suggests the existence of a slow step. When sparsomycin is preincubated with complex C before the addition of puromycin, the inhibition is increased (Fig. 1B; *two bottom lines*); this is the preincubation effect, and it can be explained by a slow equilibration between the reactive ribosomal complex and the inhibitor. This behavior can be described by the assumption that sparsomycin interacts with complex C in a two-step reaction in which the initial encounter complex CI is isomerized through a slow conformational change toward C*I:

$$C + I \underset{}{\overset{K_i}{\rightleftharpoons}} CI \underset{k_7}{\overset{k_6}{\rightleftharpoons}} C^*I \qquad (2)$$

These results expose significant differences in the kinetic behavior of the two antibiotics. The kinetic study that follows provides a detailed analysis of the mode of action of each

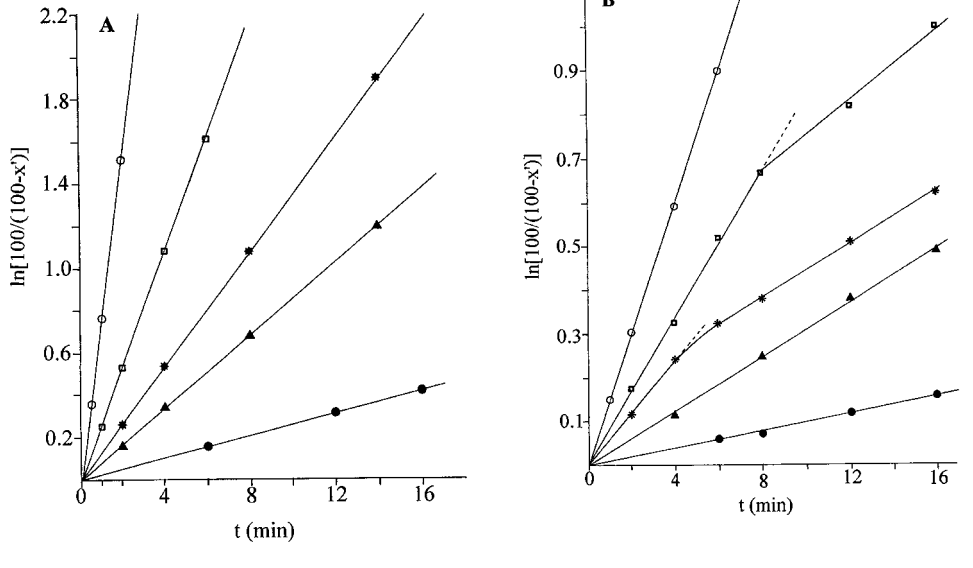

Fig. 1. First-order time plots for the reaction (A) between rabbit reticulocyte ribosomes containing complex C adsorbed on cellulose nitrate filter disks and puromycin at 4×10^{-4} M in the absence of anisomycin (○) or the presence of a mixture of puromycin and anisomycin at 1×10^{-6} M (□), 2.5×10^{-6} M (*), 5×10^{-6} M (▲), or 20×10^{-6} M (●) (B) between complex C and puromycin at 6.25×10^{-5} M in the absence of sparsomycin (○) or in the presence of a mixture of puromycin and sparsomycin at 0.1×10^{-6} M (□), and 0.2×10^{-6} M (*) or after preincubation (10 min, 25°) of complex C with sparsomycin at 0.2×10^{-6} M (▲) and 1×10^{-6} M (●) and then reaction with puromycin.

antibiotic and makes it possible to determine their differences.

Mechanism of Action of Anisomycin

For each concentration of anisomycin, there is an apparent first-order rate constant (k) that decreases with increasing concentrations of the drug. Fig. 2A shows the double-reciprocal plot of $1/k$ versus 1/[puromycin] for anisomycin concentrations ranging from 1×10^{-6} to 20×10^{-6} M. These plots are linear and they intersect, together with the plot obtained in the absence of anisomycin, at a point above the 1/[puromycin] axis. Such plots suggest that the kinetics of inhibition of peptide bond formation by anisomycin is of the mixed noncompetitive type. Dixon plots ($1/k$ versus [I]) also were found to be linear for concentrations of anisomycin up to 20×10^{-6} M (data not shown). This linearity indicates that we are dealing with complete and not partial inhibition. The slopes of the lines of Fig. 2A were plotted against the inhibitor concentration and gave a linear slope replot (not shown) from which K_i and K_s/k_3 may be determined. Finally, the intercepts of the lines of Fig. 2A with the $1/k$ axis also were plotted against the inhibitor concentration (intercept replot); this replot also was linear (Fig. 2B). The linearity of these two secondary plots suggests that the inhibition of the puromycin reaction by anisomycin follows a linear intersecting mixed noncompetitive inhibition. The linear plot of Fig. 2B meets the $1/k_{max}$ axis at a point, the reciprocal of which equals 1.67 min^{-1}. This is identical to the k_3 value of the puromycin reaction, thus confirming the mixed noncompetitive type of inhibition. A rapid attainment of equilibrium between complex C and inhibitor (I) is assumed. Under these conditions, the inhibition constant $K_i = 6.5 \times 10^{-7}$ M and $\alpha = 2$ (Fig. 2B).

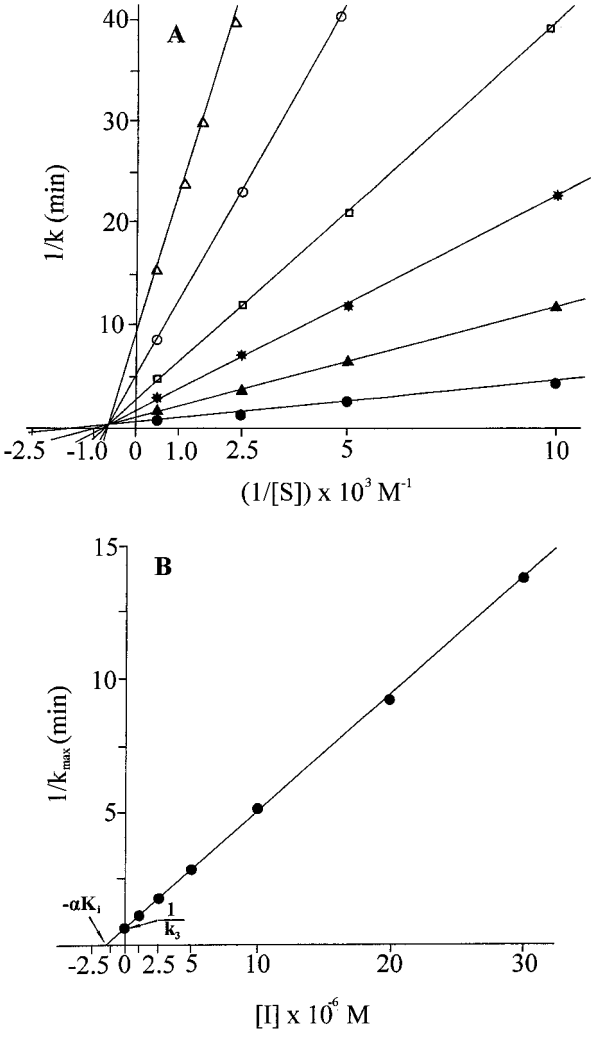

Fig. 2. A, Double-reciprocal plots ($1/k$ versus 1/[S], where S is puromycin) of the puromycin reaction (●) in the absence of anisomycin or in the presence of anisomycin at (▲) 1×10^{-6} M, (*) 2.5×10^{-6} M, (□) 5×10^{-6} M, (○) 10×10^{-6} M, and (△) 20×10^{-6} M. B, Intercept replot ($1/k_{max}$ versus [anisomycin]). The data were taken from double-reciprocal plots such as that shown in Fig. 2A. The $1/k$ axis intercepts of these plots were replotted against the concentration of anisomycin. The point on the $1/k_{max}$ axis was taken from the intercept of the line in the absence of inhibitor (control).

Mechanism of Action of Sparsomycin

In Fig. 1B, it is shown that without preincubation of eukaryotic complex C and sparsomycin, the progress curves are biphasic, whereas after preincubation, the time plots are linear. We explored these differences and studied in greater detail the mechanism of inhibition of rabbit reticulocyte peptidyltransferase by sparsomycin.

Initial slope analysis. Kinetic analysis of the initial slopes provided evidence of different types of inhibition of the puromycin reaction by sparsomycin. Thus, at several concentrations of puromycin and without preincubation with sparsomycin, the initial slopes (k) gave linear double-reciprocal plots showing competitive kinetics (Fig. 3A) from which a value of $K_i = 1.3 \times 10^{-7}$ M was obtained. By comparison, the K_i of the competitive phase in *E. coli* is 4×10^{-7} M (Kallia-Raftopoulos *et al.*, 1996). At concentrations of sparsomycin of $>0.2 \times 10^{-6}$ M, this kinetic analysis could not apply because in our system, the equilibration of the slow step occurs relatively fast, not allowing the accurate determination of the initial slope of the time plots.

If C and I are preincubated before the addition of S, the kinetics of inhibition are not competitive. As shown by the double-reciprocal plot depicted in Fig. 3B, for concentrations of I of >8 K_i, the inhibition becomes mixed noncompetitive. The intercept replot ($1/k_{max}$ versus [I]) for the entire range of inhibitor concentrations is not linear, as shown in Fig. 4. This secondary plot becomes linear for values of [I] of >8 K_i, an indication that the inhibition has already assumed its linear intersecting mixed noncompetitive character. The linear part of the plot, when extrapolated, cuts the vertical axis at 5.0 min, the reverse of which (0.2 min^{-1}) corresponds to the hypothetical k_{max} value in the absence of I. This value is much lower than the k_3 value and predicts the existence of another species that reacts with puromycin at a lower rate ($k_3^* = 0.2$ min^{-1}). The slope replot for the mixed noncompetitive phase (>8 K_i) also is linear (data not shown). The intercept of the slope replot with the vertical axis corresponds to ratio K_s^*/k_3^*, from which equilibrium constant $K_s^* = 6.4 \times 10^{-4}$ M. According to this kinetic analysis, sparsomycin exhibits an initial phase of competitive inhibition followed by a slow isomerization of CI to C*I and then by a phase of mixed noncompetitive inhibition. The latter may be explained by assuming formation of C* from C*I.

Inhibition by sparsomycin of the puromycin reaction in solution. The observation that a slow step exists in the inhibition of eukaryotic peptide bond formation by sparsomycin also was confirmed in a system in which the cellu-

lose nitrate interface is absent. Thus, when the puromycin reaction took place in solution, it was faster (k_3 = 4.55 min^{-1} and K_s = 5.9 × 10^{-4} M), but again, it displayed pseudo-first-order kinetics. Its inhibition by sparsomycin showed clearly the preincubation effect and hence confirmed the existence of a complex such as C*I as a new and modified species. A similar analysis of the initial slopes showed that this complex reacted in solution with puromycin at 25° with a rate (k_3^*) equal to 0.29 min^{-1}.

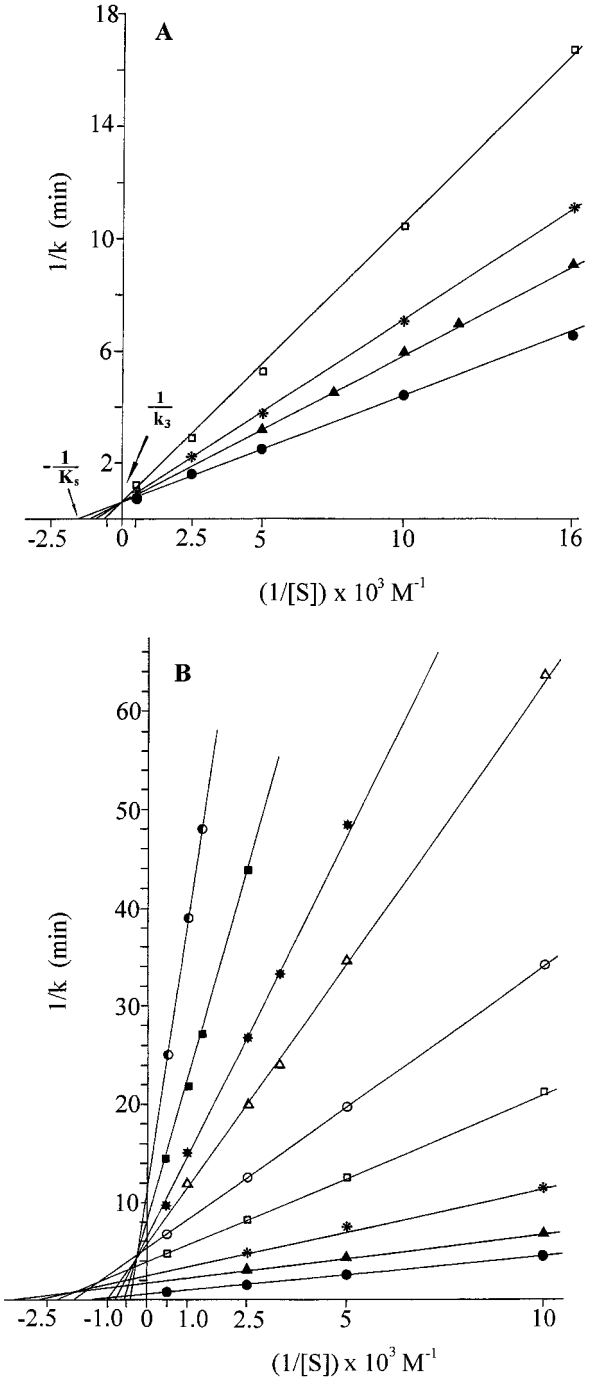

Fig. 3. A, Double-reciprocal plots (1/k versus 1/[S]) of the puromycin reaction (●) in the absence of sparsomycin or in the presence of sparsomycin at (▲) 0.05 × 10^{-6} M, (*) 0.1 × 10^{-6} M, and (□) 0.2 × 10^{-6} M. B, Double-reciprocal plots (1/k versus 1/[S]) of the puromycin reaction (●) in the absence of sparsomycin or after preincubation for 10 min at 25° of complex C with sparsomycin at (▲) 0.05 × 10^{-6} M, (*) 0.1 × 10^{-6} M, (□) 0.2 × 10^{-6} M, (○) 0.5 × 10^{-6} M, (△) 1 × 10^{-6} M, (*) 2 × 10^{-6} M, (■) 4 × 10^{-6} M, and (◐) 8 × 10^{-6} M and then reaction with puromycin.

Information obtained from the inactivation plot. To determine whether a slow step exists in the reaction between eukaryotic complex C and sparsomycin (I), the reaction of eq. 2 alone was carried out at several concentrations of I. The percentage of the remaining active complex C (x') was monitored at each time period with the puromycin reaction (2 × 10^{-3} M puromycin for 30 sec) (Fig. 5). If the inactivation process is treated as a pseudo-first-order reaction, then it approaches equilibrium with an apparent k^I_{obs} value that differs for each concentration of inhibitor. The percentage of the remaining active complex C at equilibrium is x'_{eq}. A k^I_{obs} value can be calculated for each concentration of inhibitor from the plots of $\log(x' - x'_{eq})$ versus time (Fig. 5, *inset*). The k^I_{obs} versus [I] plot gives a hyperbolic curve (not shown), which indicates that the conversion of complex C to C*I proceeds through formation of an intermediate complex CI (i.e., in two steps, similar to the situation for prokaryotes). In the absence of S, the relationship between k^I_{obs} and I predicted by the two-step mechanism is given by the equation $k^I_{obs} = k_7 + k_6 \cdot [I]/(K_i + [I])$ (Halford *et al.*, 1969; Fersht, 1985; Morrison and Walsh, 1988).

Regeneration of complex C from the sparsomycin complex C*I. To determine the rate of regeneration, the mixture of complex C and the sparsomycin complex C*I, isolated on cellulose nitrate filter disks, first was exposed to reaction buffer at 25°. At the end of each exposure, the amount of regenerated complex C was measured by reaction with puromycin (2 × 10^{-3} M for 2 min). This method gave a pseudo-first-order rate constant for sparsomycin (Fig. 6). The intercept of the straight line with the vertical axis is a measure of preexisting complex C. The slope of the line is taken as a measure of the rate of regeneration, which is analogous to the value of kinetic constant k_7. This value is equal to 0.095 min^{-1}. This low k_7 value points to the stability of the C*I complex and allowed a kinetic analysis to be made on the two-step mechanism (eq. 2 alone).

Determination of rate constant k_6. After the determination of k_7, the values of rate constant k_6 and equilibrium constant K_i can be calculated from the equation $k^I_{obs} = k_7 + k_6 \cdot [I]/(K_i + [I])$ (see Information obtained from the inactivation plot). The plot of $1/(k^I_{obs} - k_7)$ versus 1/[I] (Fig. 7) is a straight line that meets the $1/(k^I_{obs} - k_7)$ axis at a point above zero. This straight line is compatible with the two-step mechanism proposed earlier for the reaction of complex C with sparsomycin. From the plot of Fig. 7, k_6 = 2.1 min^{-1} and K_i = 2.2 × 10^{-7} M. This K_i value is close to 1.3 ×10^{-7} M, as determined previously from the competitive phase of the inhibition of eukaryotic peptidyltransferase by sparsomycin.

The individual values of k_6 and k_7 combined with the fact that ratio k_6/k_7 = 22 permit the characterization of sparsomycin as a slow-binding inhibitor of rabbit reticulocyte peptidyltransferase.

Discussion

The current study is an attempt to examine the inhibition of ribosomal peptidyltransferase from eukaryotic cells. For this purpose, we used a recently developed *in vitro* system for the determination of the activity status of peptidyltransferase from rabbit reticulocyte ribosomes (Ioannou *et al.*,

1997). In this system, preformed complex C, containing rabbit reticulocyte ribosomes, AcPhe-tRNA from *E. coli*, and poly(U), reacts with excess puromycin and catalyzes in a pseudo-first-order reaction, the formation of peptide bonds in AcPhe-puromycin. The study of the kinetics of inhibition of this reaction by several antibiotics provides relevant information on ribosomal structure and function. In this case, two drugs with different cell specificities were chosen, anisomycin and sparsomycin.The former initially was described as inhibitor of eukaryotic peptidyltransferase and later was found also to be an inhibitor of archaebacterial peptidyltransferase, whereas sparsomycin is a universal inhibitor of peptidyltransferase. The primary aim of this study was to compare and contrast, for a given organism, the mechanism of inhibition displayed by these two antibiotics.

The antibiotics examined up until now initially had been described as classic inhibitors of *E. coli* peptidyltransferase. However, under the kinetic treatment described in this and previous reports (Kallia-Raftopoulos *et al.*, 1992, 1996; Theocharis *et al.*, 1992; Dinos *et al.*, 1993), several were found to cause time-dependent inhibition involving a conformational change during the slow isomerization of encounter complex CI to C*I before further reaction with S. The question then arose of whether there are antibiotics-inhibitors of ribosomal peptidyltransferase that do not behave in our kinetic analysis as slow-binding inhibitors. As clearly shown in this re-

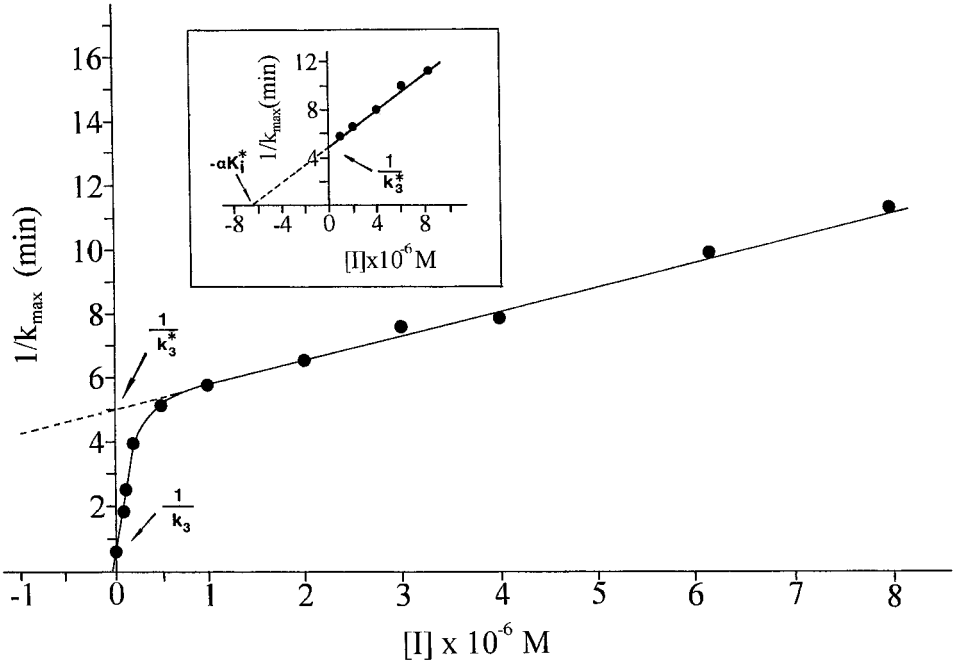

Fig. 4. Intercept replot ($1/k_{max}$ versus [sparsomycin]). After preincubation of complex C with various concentrations of sparsomycin, the $1/k$ axis intercepts ($1/k_{max}$) of the double-reciprocal plots of Fig. 3B were replotted against the concentrations of sparsomycin. *Inset*, detail of Fig. 4 from which k_3^* and K_i^* can be accurately determined.

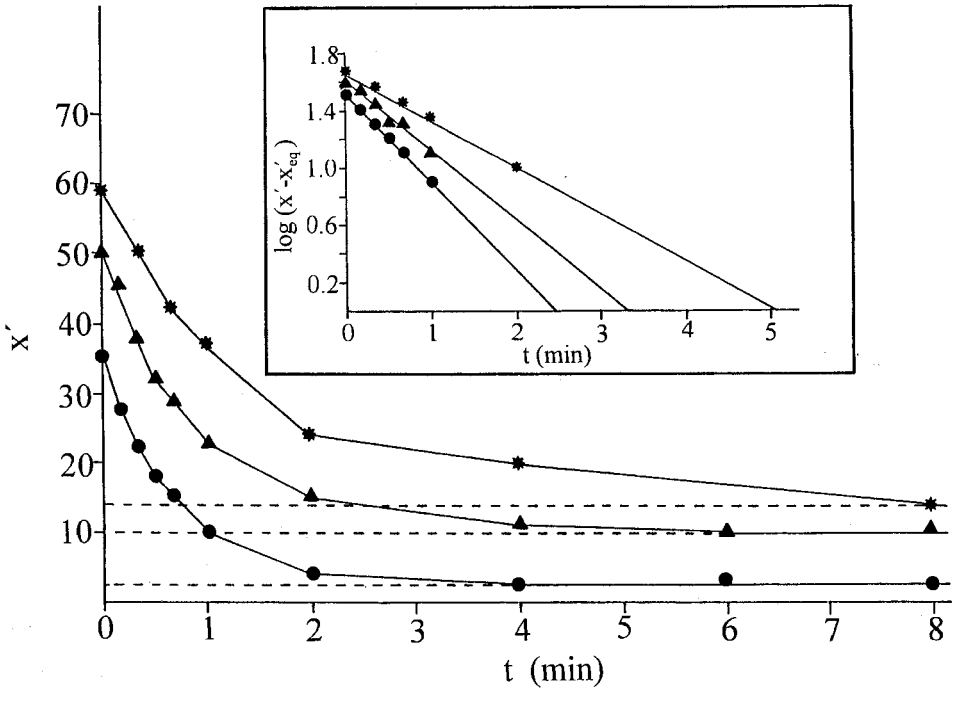

Fig. 5. Time plots for the inactivation of eukaryotic complex C by sparsomycin. Complex C adsorbed on cellulose nitrate filter disks reacted for the indicated time intervals with sparsomycin at (*) 0.1×10^{-6} M, (▲) 0.2×10^{-6} M, and (●) 0.4×10^{-6} M. The percentage (x') of the remaining active complex C is estimated through titration with puromycin at 2×10^{-3} M for 30 sec. *Inset*, plot of $\log(x' - x'_{eq})$ versus time from which a k^I_{obs} value for each concentration of sparsomycin can be calculated.

port, anisomycin is one such antibiotic whose behavior is far from being classified as slow binding. Our results suggest that anisomycin behaves as a classic mixed noncompetitive inhibitor (Figs. 1A and 2) with a K_i value of 6.5×10^{-7} M. The product, AcPhe-puromycin, is derived only from CS with a k_3 value of 1.67 min^{-1} according to the puromycin reaction. Previous reports have shown that the drug partially prevented the binding of both donor and acceptor substrates to the ribosomal peptidyltransferase center (Battaner and Vazquez, 1971) or that it inhibited the binding of AcPhe-tRNA and Phe-tRNA into P and A site, respectively (Carrasco and Vazquez, 1972). Thus, despite the fact that anisomycin inhibited competitively the puromycin reaction on native polyribosomes (Pestka *et al*., 1972), it is by no means evident that the action of the drug is exerted exclusively at the ribosomal A site (Gale *et al*., 1981). Recently, anisomycin was classified as both an A site and an E site inhibitor (Rodriguez-Fonseca *et al*., 1995). Anisomycin has two groups important for its activity: the basic pyrrolidin ring is required for its activity because either acetylation of the nitrogen atom

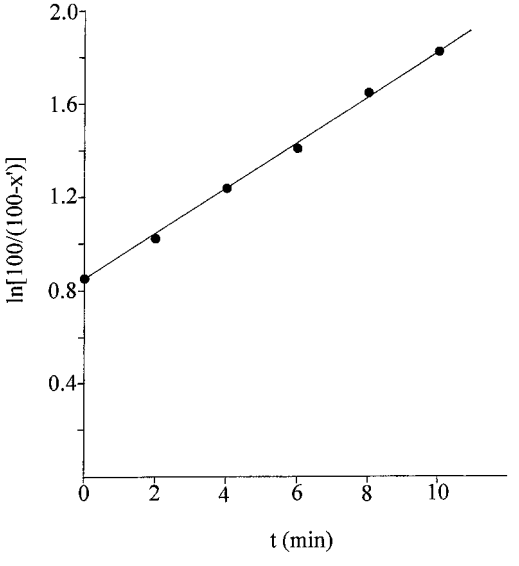

Fig. 6. Determination of the pseudo-first-order rate constant for the regeneration of complex C from the sparsomycin complex C*I after exposure to reaction buffer for several time intervals and reaction with 2×10^{-3} M puromycin for 2 min.

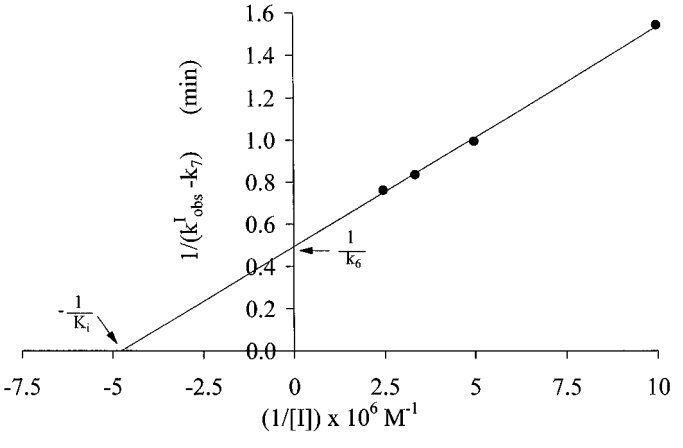

Fig. 7. Double-reciprocal plot ($1/[k^I_{obs} - k_7]$ versus 1/[sparsomycin]) for the inactivation of complex C by sparsomycin. The k^I_{obs} values for each concentration of sparsomycin were taken from Fig. 5 (*inset*).

or deacetylation of the 3′ position renders the molecule inactive. Similar effects are produced by bromination of the *p*-methoxyphenyl moiety. Moreover, nucleotides that are altered in the presence of anisomycin within the peptidyltransferase loop region of domain V of 23S-like rRNA seem to belong to at least two distinct subsites: one group of nucleotides belongs to a region near the "catalytic subsite," whereas the second group to the subsite is assigned to the entrance to peptide channel. This fact suggests the possibility of an allosteric effect by anisomycin, which would be compatible with the mixed noncompetitive type of inhibition that we find.

Sparsomycin, on the other hand, is fully active in all cell types; therefore, it represents a unique opportunity to conclude the study of inhibition of both prokaryotic and eukaryotic peptidyltransferase and compare the kinetics of inhibition of each of them by the same antibiotic. Significantly, sparsomycin behaves in rabbit reticulocyte ribosomes in the same way as in *E. coli* ribosomes: there is an initial, rapid reaction of the drug with complex C, which is characterized by competitive kinetics before the isomerization of CI (Fig. 3A), followed by a mixed noncompetitive phase after the isomerization of CI to C*I and at drug concentrations >8 K_i (Fig. 3B). In this phase, product is received from a new, modified ribosomal complex at a reduced rate, k^*_3. More importantly, sparsomycin, just like in prokaryotic cells, seems to react in a time-dependent manner (Figs. 3B and 4) and induces conformational changes in CI, which is isomerized to C*I. Identical kinetic behavior of sparsomycin was observed when the puromycin reaction took place in solution, in which possible artifacts from the cellulose nitrate interface are avoided. It may be assumed that C*I is converted to C* before it reacts with S at a reduced rate to produce AcPhe-puromycin.

Sparsomycin fulfills the criteria for its characterization as a slow-binding inhibitor [i.e., biphasic progress curves (Fig. 1B), the preincubation effect (Figs. 1B and 3B) and the shape of inactivation plots (Figs. 5 and 7)], all of which obviously are missing from the reaction of anisomycin with complex C. Moreover, our analysis permitted the determination of rate constants rather than of equilibrium constants such as K_i, which cannot by itself represent the potency of the inhibitor at the late phase of inhibition. After determination of k_7 (Fig. 6), an apparent association rate constant (Schloss, 1988) for sparsomycin and eukaryotic complex C can be calculated. This is equal to $k_7/K_i' = 2 \times 10^5$ M^{-1} sec^{-1}, where $K_i' = K_i (k_7/k_6 + k_7)$. Information on such constants is not widely available in the field of inhibitors of peptide bond formation. In fact, association rate constants have been reported previously only for free ribosomes and antibiotics such as spiramycin, lincomycin, or erythromycin (DiGiambattista *et al*., 1987). Our apparent association rate constant is a more accurate measure of the potency of an antibiotic. By comparison, in *E. coli*, $k_7/K_i' = 1 \times 10^5$ M^{-1} sec^{-1} (Dinos *et al*., 1993). Thus, on the basis of its similar association rate constants, sparsomycin can be characterized as an equally potent inhibitor of peptide bond formation in both eukaryotes and prokaryotes. These results add kinetic evidence to the notion that sparsomycin is a universal inhibitor of ribosomal peptidyltransferase.

Studies from this laboratory on sparsomycin and other inhibitors of peptidyltransferase combined with data on

rRNA footprints for the different antibiotics and on ribosomal ligand binding prompted Kirillov *et al*. (1997) to make some inferences about the catalytic center. Thus, the drugs may bind initially at a site through which the acceptor end of aminoacyl-tRNA passes after its release from the ternary complex and before peptide bond formation. In this state, each drug can bind competitively with the acceptor substrate and with other drugs. The subsequent slow change that occurs may correspond to a drug-induced change in the conformation of the 23S rRNA, possibly involving an increased opening or accessibility of the catalytic center and thereby producing an inactive ribosome and noncompetitive kinetics of drug binding. Besides sparsomycin, this class of inhibitors would include chloramphenicol, blasticidin S, and amicetin but not anisomycin. Other studies have implied that anisomycin is a poor competitor of the sparsomycin interaction with the eukaryotic ribosome. Thus, in yeast ribosomes, anisomycin is not a good competitor of sparsomycin interaction (Barbacid and Vazquez, 1974a; Lazaro *et al*., 1991b), whereas in human tonsil ribosomes, there is no competition between these two antibiotics (Barbacid and Vazquez, 1974a). These results lend credence to the notion put forward by Barbacid and Vazquez (1974b) that antibiotics acting universally, such as sparsomycin, bind to a structural part of the peptidyltransferase center that is common in both prokaryotic and eukaryotic ribosomes. Antibiotics acting on eukaryotic ribosomes only, such as anisomycin, bind to another structural part of this center, which is different in prokaryotic and eukaryotic ribosomes.

In conclusion, the results of the current study provide kinetic evidence that anisomycin and sparsomycin exhibit different inhibitory mechanisms of peptide bond formation in eukaryotes. This seems to indicate that largely different sites of the peptidyltransferase center are involved in the binding of these two peptidyltransferase inhibitors.

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