Interaction between the Antibiotic Spiramycin and a Ribosomal Complex Active in Peptide Bond Formation†

George Dinos, Dennis Synetos, and Charalambos Coutsogeorgopoulos*

Laboratory of Biochemistry, School of Medicine, University of Patras, 261 10 Patras, Greece

Received March 8, 1993; Revised Manuscript Received June 23, 1993®

ABSTRACT: The inhibition of peptide bond formation by spiramycin was studied in an in vitro system derived from *Escherichia coli*. Peptide bonds are formed between puromycin (S) and Ac-Phe-tRNA, which is a component of complex C, i.e., of the [Ac-Phe-tRNA–70S ribosome–poly(U)] complex, according to the puromycin reaction: $C + S\ (K_s) \rightleftharpoons CS\ (k_3) \rightarrow C' + P$ [Synetos, D., & Coutsogeorgopoulos, C. (1987) *Biochim. Biophys. Acta 923*, 275–285]. It is shown that spiramycin (A) reacts with complex C and forms the spiramycin complex C*A, which is inactive toward puromycin. C*A is the tightest complex formed between complex C and any of a number of antibiotics, such as chloramphenicol, blasticidin S, lincomycin, or sparsomycin. C*A remains stable following gel chromatography on Sephadex G-200 and sucrose gradient ultracentrifugation. Detailed kinetic study suggests that C*A is formed in a variation of a two-step mechanism in which the initial encounter complex CA is kinetically insignificant and C*A is the product of a conformational change of complex CA according to the equation, $C + A\ (k_{assoc}) \rightleftarrows (k_{dissoc})\ C^*A$. The rate constants of this reaction (spiramycin reaction) are $k_{assoc} = 3.0 \times 10^4\ M^{-1}\ s^{-1}$ and $k_{dissoc} = 5.0 \times 10^{-5}\ s^{-1}$. Such values allow the classification of spiramycin as a slow-binding, slowly reversible inhibitor; they also lead to the calculation of an apparent overall dissociation constant equal to 1.8 nM for the C*A complex. Furthermore, they render spiramycin a useful tool in the study of antibiotic action on protein synthesis in vitro. Thus, the spiramycin reaction, in conjunction with the puromycin reaction, is applied (i) to detect a strong preincubation effect exerted by chloramphenicol and lincomycin (this effect constitutes further evidence that these two antibiotics combine with complex C as slow-binding inhibitors) and (ii) to determine the rate constant for the regeneration ($k_7 = 2.0 \times 10^{-3}\ s^{-1}$) of complex C from the sparsomycin complex C*I [Theocharis, D. A., & Coutsogeorgopoulos, C. (1992) *Biochemistry 31*, 5861–5868] according to the equation, $C + I\ (K_i) \rightleftharpoons CI\ (k_6) \rightleftharpoons (k_7)\ C^*I$. The determination of k_7 enables us to calculate the apparent association rate constant of sparsomycin, $(k_7/K_{i'}) = 1.0 \times 10^5\ M^{-1}\ s^{-1}$, where $K_{i'} = K_i(k_7/k_6 + k_7)$. It is also shown that Ac-Phe-tRNA bound to the sparsomycin complex C*I is protected against attack by hydroxylamine. In contrast, Ac-Phe-tRNA bound to spiramycin complex C*A is susceptible to hydroxylaminolysis; the pseudo-first-order rate constant at 0.4 M aqueous hydroxylamine is equal to $5.2 \times 10^{-4}\ s^{-1}$ for Ac-Phe-tRNA bound to C*A, whereas it is much lower for Ac-Phe-tRNA bound to C*I ($4.8 \times 10^{-5}\ s^{-1}$).

Studies on ribosomal structure and function have been greatly aided by the use of antibiotics acting as inhibitors of protein synthesis. Such studies have led to information on the functional domains of the ribosome or on antibiotic binding sites (Cundliffe, 1987; Cooperman, 1987; Hall et al., 1988; DiGiambattista et al., 1990). Prominent among these antibiotics are the macrolides. Spiramycin is one of them, and it has been found to inhibit peptide bond formation on bacterial ribosomes (Vazquez, 1979). The studies with spiramycin have been conducted either as binding studies on free ribosomes (Pestka, 1974; DiGiambattista et al., 1987; Tejedor & Ballesta, 1986) or as inhibition studies on polypeptide-synthesizing systems (Vazquez, 1967). We have employed a ribosomal complex, the [Ac-Phe-tRNA-70S ribosome–poly(U)] complex (complex C), in which the donor Ac-Phe-tRNA is reactive toward puromycin and forms peptide bonds. This complex has been used widely in protein synthesis studies [recent references include Moazed and Noller (1989), Odom et al. (1991), and Odom and Hardesty (1992)]. The reaction of complex C with excess puromycin can be conveniently analyzed as a pseudo-first-order reaction (Synetos & Coutsogeorgopoulos, 1987, 1989).

In the past few years, we have carried out detailed studies on the inhibition of the puromycin reaction by several antibiotics. These studies provided evidence that the antibiotics chloramphenicol, blasticidin S (Theocharis et al., 1992), lincomycin (Kallia-Raftopoulos et al., 1992), and sparsomycin (Theocharis & Coutsogeorgopoulos, 1992) do not behave as classical reversible inhibitors, but rather they fall into the class of slow-binding inhibitors (Williams & Morrison, 1979). In addition, the inhibitor (I) blocks peptide bond formation between puromycin and the Ac-Phe-tRNA of complex C in a mechanism where the familiar intermediate complex (CI) undergoes conformational changes and gives rise to a new species, defined as C*I. These changes are detected as transitions from one type of inhibition to another, i.e., from competitive to mixed noncompetitive (Theocharis et al., 1992). Recently, Marconi et al. (1990), using different methodology, presented evidence that conformational alterations in rRNA structure may be the basis for the inhibitory effect of chloramphenicol on protein biosynthesis. There is scope for further studies on the molecular basis of the conformational changes occurring (i) during the interaction of puromycin with complex C (Moazed & Noller, 1989; Odom & Hardesty, 1992) or with a similar complex (Odom & Hardesty, 1987)

† This work was supported in part by a grant from the General Secretariat of Research and Technology, Ministry of Industry, Energy and Technology of Greece.

® Abstract published in *Advance ACS Abstracts,* August 15, 1993.

0006-2960/93/0432-10638$04.00/0

and (ii) during the interaction of complex C with various antibiotics.

In the present study, we have investigated the interaction between complex C and spiramycin. We find that spiramycin exhibits a distinctive pattern of inhibition of peptide bond formation and forms the tightest complex that can be produced between complex C and any of the other inhibitors of peptide bond formation we have studied so far, i.e., chloramphenicol, blasticidin S, lincomycin, and sparsomycin.

MATERIALS AND METHODS

Materials

L-Phenylalanine, poly(U), GTP (disodium salt), ATP (disodium salt), puromycin dihydrochloride, and transfer ribonucleic acid from *Escherichia coli* strain W were purchased from Sigma Chemical Co. (U.K.). The spiramycin was a mixture of spiramycins I, II, and III and, it was also purchased from Sigma. Sodium borohydride was from Serva Feinbiochemica (Germany). Sodium [^{3}H]borohydride and L-phenyl-[2,3-$^3H_2$]alanine were purchased from Amersham (U.K.). Sephadex G-200 was from Pharmacia Fine Chemicals (Sweden). Zwittergent 3-12 (ZW) detergent (*N*-dodecyl-*N*,*N*-dimethyl-3-ammonium-1-propanesulfonate) was obtained from Calbiochem AG. Cellulose nitrate filterdisks (type HA, 24 mm diameter, 0.45 μm pore size) were purchased from Millipore Corporation. Silica gel 60 F_{254} precoated sheets (layer thicknesses 0.2 and 2 mm for analytical and preparative TLC, respectively) were from Merck (Germany).

Methods

Preparation of Complex C. Complex C, the Ac-[^{3}H]Phe-tRNA–70S ribosome–poly(U) complex, was formed in a binding mixture which was prepared at 0 °C by adding the following (per 0.2 mL): 20 μmol of Tris-HCl (pH 7.2), 20 μmol of NH_4Cl from a solution adjusted with NH_4OH to pH 7.2, 2 μmol of magnesium acetate, 64 μg of poly(U), 0.08 μmol of GTP, 6.4 A_{260} units of washed ribosomes (Synetos & Coutsogeorgopoulos, 1987), 80 μg of (protein) ribosomal wash (FWR fraction), and 65 pmol of Ac-[^{3}H]Phe-tRNA (2000 cpm/pmol) or, when required, Ac-[^{14}C]Phe-tRNA (500 cpm/pmol). After incubation at 25 °C for 8 min, the binding mixture was placed in ice, and complex C was separated from excess donor Ac-Phe-tRNA as well as from other molecules present in the binding mixture by dilution with ice-cold binding buffer [100 mM Tris-HCl (pH 7.2), 50 mM KCl, 10 mM $MgCl_2$, and 6 mM β-mercaptoethanol], filtration through cellulose nitrate disks, and three washes with binding buffer. When required, complex C was desorbed into a solution containing the detergent Zwittergent 3-12 (ZW extract) according to a method described earlier (Theocharis & Coutsogeorgopoulos, 1989).

Puromycin Reaction. The puromycin reaction was employed for two distinct purposes: (i) to measure the activity of peptidyltransferase in complex C via determination of a k_{cat} and K_s as described elsewhere (Theocharis & Coutsogeorgopoulos, 1989). Briefly, this was done by allowing complex C, either on a cellulose nitrate disk or in solution, to react with an excess of puromycin and by analyzing the progress of the reaction over a wide range of puromycin concentrations. The second purpose was (ii) to titrate the amount of active complex C either after its reaction with spiramycin (spiramycin reaction) or after its regeneration from the spiramycin (C*A) or sparsomycin (C*I) complexes. The amount of active complex C was monitored by reaction with 2 mM puromycin ($5K_s$) for 2 min (7 half-lives).

Inactivation of Complex C by Spiramycin (Spiramycin Reaction). We call the nearly irreversible interaction between spiramycin (A) and complex C (C) the spiramycin reaction. The resulting complex C*A is called the spiramycin complex. This reaction was carried out essentially as described in Coutsogeorgopoulos et al. (1975). Complex C, either adsorbed on cellulose nitrate disks or desorbed from the disks into a solution (ZW extract), reacted with various concentrations of spiramycin in 1 mL of reaction buffer [100 mM Tris-HCl (pH 7.2), 100 mM NH_4Cl, 10 mM $MgCl_2$, and 6 mM β-mercaptoethanol]. The reaction was allowed to proceed at 25 °C for the desired time intervals and was stopped by dilution with 15 mL of cold binding buffer. Following filtration and two washes with 4 mL of the same buffer, complex C isolated on the disk in a mixture with C*A was reacted with 2 mM puromycin for 2 min. For each concentration of spiramycin, the log of the percent x' of complex C that reacted with puromycin was determined and plotted against time, assuming that the reaction between complex C and spiramycin proceeded as a pseudo-first-order reaction. The inactivation of complex C by spiramycin was also examined in the presence of the antibiotics lincomycin or chloramphenicol. Alternatively, complex C was first exposed to chloramphenicol or lincomycin for the specified periods of time (stage of preincubation). Subsequently, spiramycin was added and allowed to react for the indicated time intervals. In both cases, the reactions were stopped by dilution with 15 mL of cold binding buffer and the inactivation was followed as mentioned above (2 mM puromycin, 2 min). These experiments were also performed with a spiramycin derivative, dihydrospiramycin, used in place of spiramycin.

The spiramycin complex C*A can be prepared not only via the spiramycin reaction but also by including spiramycin (usually 1×10^{-5} or 1×10^{-6} M) in the binding mixture. In either case, the ratio [C*A]:[C] depends on the concentration of spiramycin and the time interval used.

*Regeneration of Complex C from the Spiramycin Complex C*A.* The rate of regeneration of complex C was determined in two ways: (a) Following the binding reaction, the excess spiramycin was removed by filtration through cellulose nitrate disks, after which the disk was immersed in 1 mL of reaction buffer containing 2 mM puromycin. Ac-[^{3}H]Phe-puromycin was then measured for periods of incubation of up to 64 min. (b) The binding mixture was applied on a Sephadex G-200 column (details under Gel Chromatography), and the eluted spiramycin complex C*A was exposed to binding buffer at 25 °C for up to 64 min. The recovery of complex C from C*A was subsequently monitored by reaction with 2 mM puromycin for 2 min.

Reaction of Spiramycin with Complex C Regenerated from the Sparsomycin Complex. The inactivation experiments were also carried out when complex C on the disk was replaced by a mixture of complex C and sparsomycin complex C*I (Theocharis & Coutsogeorgopoulos, 1992). Briefly, this mixture was formed when complex C was prepared in the presence of 1.0 μM sparsomycin in the binding mixture. Following filtration on cellulose nitrate disks, the mixture of complex C and sparsomycin complex C*I was exposed to spiramycin for several time intervals, and the process was monitored using the puromycin reaction (2 mM, 2 min).

Regeneration of Complex C from the Sparsomycin Complex. The mixture of complex C and sparsomycin complex C*I on cellulose nitrate disks was exposed to binding buffer

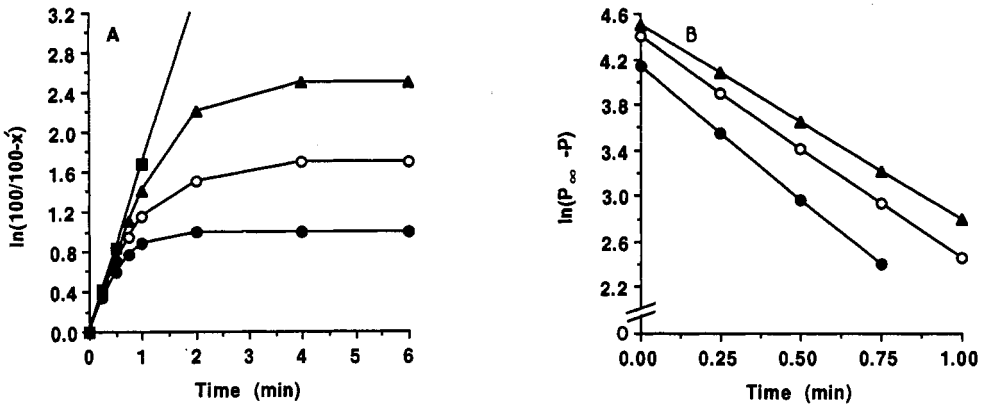


FIGURE 1: (A) First-order time plots for Ac-Phe-puromycin formation. Complex C in solution (ZW extract) reacts with (■) 0.4 mM puromycin (control) or with 0.4 mM puromycin in the presence of spiramycin at the following concentrations: (▲) 0.2 μM; (○) 0.5 μM; (●) 1.0 μM. x' is defined in Theocharis and Coutsogeorgopoulos (1989). (B) Determination of the apparent rate constant F after replotting the data of A in the form of ln $(P_\infty - P)$ versus time.

for various periods of time. Subsequently, the disk was removed, placed in cold binding buffer, filtered, and washed with the same buffer. The regeneration of complex C from C*I was monitored using the puromycin reaction (2 mM puromycin, 2 min).

Gel Chromatography. Gel chromatography was carried out in a column (70 × 0.84 cm) packed with Sephadex G-200 and equilibrated with binding buffer at 4 °C. Binding mixture, usually 0.2 mL, prepared in the absence or presence of spiramycin was applied on the column.

The column was eluted at 4 °C with binding buffer; 1.0-mL fractions were collected, and their radioactivity was monitored in a liquid scintillation spectrometer. The fractions containing the peak of radioactivity in the void volume were pooled and divided into an appropriate number of aliquots. Each aliquot was passed through a cellulose nitrate disk and washed with ice-cold binding buffer. Thus, complex C and/or the spiramycin complex were isolated on cellulose nitrate disks. When required, C*A and/or C were desorbed into a solution as described under Preparation of Complex C (ZW extract), and the ZW extract (0.8 mL) was reapplied to the G-200 column for a second run performed as above.

Preparation of Dihydrospiramycin and [^3H]Dihydrospiramycin. This was performed accoding to Tejedor and Ballesta (1985) with some modifications. Briefly, 32.6 μmol (30 mg) of spiramycin base (MW taken as equal to 876) was dissolved in 306 μL of diethylene glycol dimethyl ether and mixed with 29.5 μmol of $NaBH_4$ dissolved in 75 μL of the same solvent. The mixture was shaken at room temperature for 120 min. Then, 1.5 mL of 0.5 M $NaHCO_3$ (pH 9.35) was added, and the mixture was shaken in an ice bath for 30 min and extracted three times with 2 mL of methylene chloride. After washing with water until neutrality, the organic phase was concentrated by evaporation at room temperature. The extent of the reduction was checked by analytical thin-layer chromatography (TLC) on silica gel and eluted with a mixture of chloroform and methanol (8:1). The final product was recovered by preparative TLC under the same elution conditions, yielding 22 mg of dihydrospiramycin. The purity of the product was monitored by the orcinol reaction as well as by infrared spectroscopy. Radioactive dihydrospiramycin was prepared in the same way from a mixture of tritiated $NaBH_4$ (10 mCi, 9.5 μmol) and unlabeled $NaBH_4$ (20 μmol).

Reaction of Hydroxylamine with the Donor Ac-Phe-tRNA. When Ac-Phe-tRNA was bound to complex C, its reaction with hydroxylamine was followed out in the ZW extract for the indicated time intervals. Filtration of the solution through a cellulose nitrate disk followed, upon which the non-reacted complex C was retained on the filter while the product of the reaction, Ac[^{3}H]Phe-hydroxamate, passed through. The decrease in radioactivity is a measure of the bound Ac-Phe-tRNA that reacted with hydroxylamine.

In further experiments, donor Ac-Phe-tRNA bound to complex C (ZW extract) was first exposed at 25 °C for 5 min to one of several antibiotics, including spiramycin (1×10^{-5} M), lincomycin (1×10^{-4} M), chloramphenicol (5×10^{-5} M), sparsomycin (1×10^{-6} M), or blasticidin S (1×10^{-5} M). Under these conditions there is equilibration between free C and species that contain C as well as the antibiotic. Reaction with hydroxylamine was followed at 25 °C for various time intervals, and the reaction was monitored as described in the previous paragraph.

As a control, the reaction of free Ac-[^{3}H]Phe-tRNA with hydroxylamine was carried out at 25 °C for the indicated time intervals, and the amount of Ac-Phe-tRNA that reacted was estimated from the reduction in trichloroacetic acid precipitable radioactivity assayed with Whatman 3MM filter paper disks.

RESULTS

Inhibition of Peptide Bond Formation by Spiramycin. The inhibition of peptide bond formation by spiramycin was studied in a model system in which the ternary complex, Ac-Phe-tRNA–70S ribosome–poly(U) (complex C), was isolated on cellulose nitrate disks free of excess unbound Ac-Phe-tRNA. It then reacted either on the disks or in solution (ZW extract) with excess puromycin (S) according to the ribosome-catalyzed reaction shown in eq 1 (Synetos & Coutsogeorgopoulos, 1987).

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

A peptide bond is formed in Ac-Phe-puromycin (P), the determination of which monitors the progress of the reaction; the C′ form cannot revert back to C. Thus, puromycin acts as a pseudosubstrate in this reaction, which could also represent the irreversible inactivation of complex C by puromycin.

Figure 1A depicts the time course of the reaction between complex C (ZW extract) and 4×10^{-4} M puromycin in the absence or presence of increasing concentrations of spiramycin (A). In the absence of spiramycin, a straight line is obtained from the logarithmic plot until all of complex C has been converted to product Ac-Phe-puromycin. The reaction is over in less than 3 min. In the presence of spiramycin the reaction

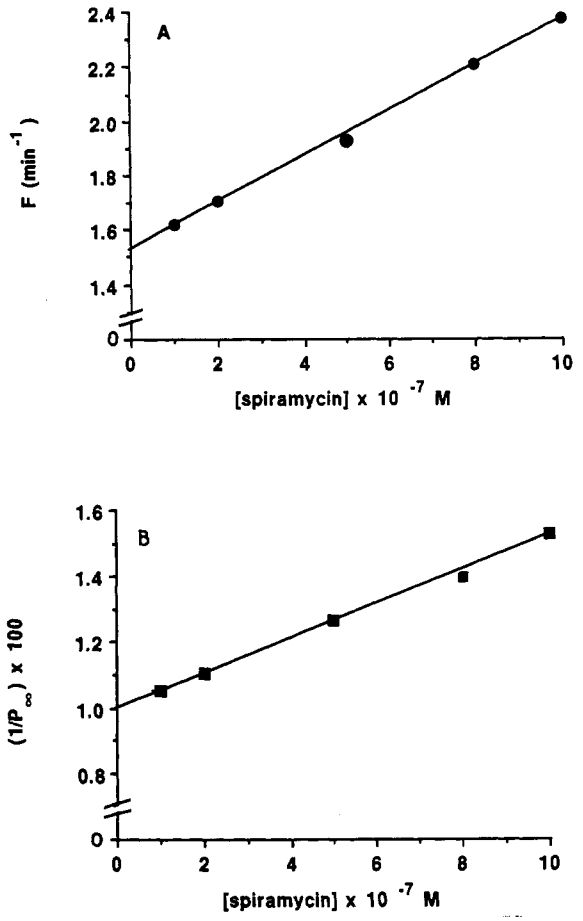


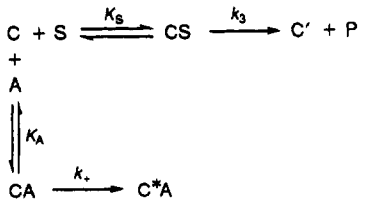
FIGURE 2: (A) Variation of the apparent rate constant F as a function of spiramycin concentration. (B) Plot of $1/P_\infty$ versus spiramycin concentration. The F and P_∞ values were determined according to the experimental conditions of Figure 1.

is slower, and finally less complex C reacts with puromycin; each progress curve reaches a plateau given by ln (100/100 $-x'_\infty$), where x'_∞ is the upper limit of x'. x' equals x/a, where a is a correction factor defined in Theocharis and Coutsogeorgopoulos (1989); x' represents the percentage of complex C converted to product P. x'_∞ decreases with increasing concentrations of spiramycin. These results indicate that spiramycin inhibits product formation rather irreversibly.

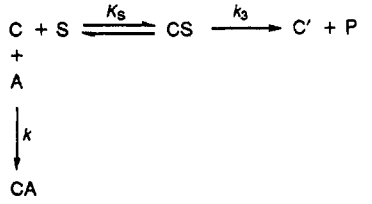
If at each concentration of spiramycin we substitute ln ($x'_\infty - x'$) by its equivalent ln ($P_\infty - P$), we obtain the plot of ln($P_\infty - P$) versus time which is shown in Figure 1B. The slopes of the straight lines thus obtained give the values of an apparent rate constant F. This constant corresponds to the simultaneous inactivation of complex C by puromycin and spiramycin, which proceeds as a pseudo-first-order reaction. When F was plotted against various concentrations of spiramycin, a straight line was obtained (Figure 2A). Similarly, as shown in Figure 2B, the $1/P_\infty$ versus [A] plot shows that increasing concentrations of spiramycin result in lower values of P_∞, and this is compatible with the assumptions that C is inactivated by two parallel first-order reactions and that spiramycin and puromycin compete with each other for complex C. The same pattern of results was obtained when complex C reacted while adsorbed on cellulose nitrate disks.

The theoretical derivation of the expressions which give the apparent pseudo-first-order rate constant F, as well as factor G, for two possible kinetic schemes (Schemes I and II) are given in the Appendix. In these schemes, C is complex C, S is puromycin, and A is spiramycin.

Scheme I



Scheme II



These schemes have been derived on the basis of the assumption that spiramycin is an irreversible inhibitor. Scheme I represents a two-step mechanism for the reaction between C and A, whereas Scheme II describes a one-step mechanism (Tian & Tsou, 1982; Leytus et al., 1984). An attempt to analyze Scheme I for a reversible inhibitor, i.e., when k_- is not zero, has been presented elsewhere (Kallia-Raftopoulos et al., 1992).

We carried out experiments similar to those of Figure 1, but with varying concentrations of puromycin at a constant concentration of spiramycin (2×10^{-6} or 4×10^{-6} M). The form of the time plots (not shown) was similar to the three lower lines of Figure 1A. The plot of $1/F$ versus [S] (Figure 3A) shows that the value of the constant F depends on the concentration of puromycin as well. This plot is not linear; however, the plot of $1/P_\infty$ versus $1/[S]$ is linear (Figure 3B). The fact that the plots of F versus [A] (Figure 2A), $1/P_\infty$ versus [A] (Figure 2B), and $1/P_\infty$ versus $1/[S]$ (Figure 3B) are linear supports the one-step mechanism as predicted by eqs A.2 and A.9 (substitute F from A.2 and G from A.6) of the Appendix. These plots would not be linear for the two-step mechanism as predicted by eqs A.3 and A.9 (substitute F from A.3 and G from A.7) (Appendix). Moreover, G is independent of [A], i.e., the plot of G versus [A] (not shown) is a line parallel to the [A] axis, and this fact is also compatible with the one-step mechanism according to eq A.6 (Appendix). In the case of the two-step mechanism, G depends on [A] according to eq A.7. From the slopes of the plots F versus [A] and $1/P_\infty$ versus [A], the rate constant $k = 3.3 \times 10^4$ M^{-1} s^{-1} (Scheme II) can be calculated using eqs A.2 and A.9 (substitute F from A.2 and G from A.6) for the one-step mechanism. Furthermore, the results depicted in Figures 2 and 3 indicate that puromycin and spiramycin react with C in a competitive way, i.e., there is formation of either CS or CA but not CAS (Schemes I and II); this competition, however, cannot be expressed by the classical competitive plots obtained for reversible enzyme inhibitors.

Inactivation of Complex C by Spiramycin (Spiramycin Reaction). The reaction of complex C with spiramycin in the absence of puromycin, i.e., the inactivation of complex C by spiramycin alone, was examined by exposing complex C to spiramycin for several time intervals and monitoring the remaining active complex C by reaction with puromycin. These inactivation plots are shown in Figure 4A, in which the plot of log x' versus time gives a straight line for each concentration of spiramycin, showing that the inactivation follows pseudo-first-order kinetics. For each concentration of spiramycin, we obtain a pseudo-first-order rate constant of inactivation

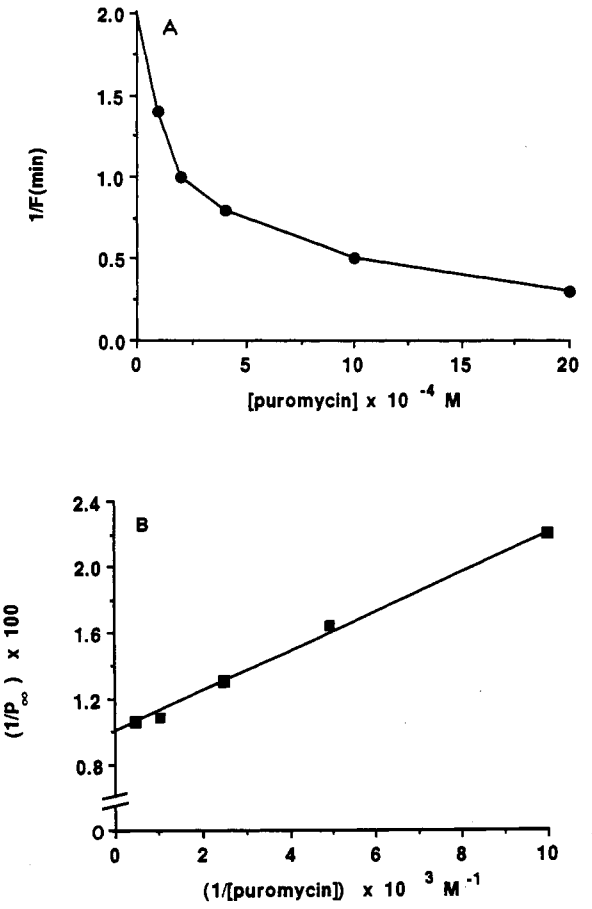


FIGURE 3: (A) Dependence of the reciprocal of rate constant F on the puromycin concentration. (B) Dependence of the reciprocal of P_∞ on the reciprocal of puromycin concentration. Experiments were performed as described in Methods with complex C on cellulose nitrate disks. The concentration of spiramycin was 2×10^{-6} M.

(k_{in}) whose reciprocal, if plotted against the reciprocal of the concentration of spiramycin, gives a straight line that meets the $1/k_{in}$ axis at a point above zero (Figure 4B). This would indicate that the conversion of complex C to a form (C*A) which is inactive toward puromycin proceeds via formation of an encounter complex CA, implying the reactions of eq 2. However, over the range of inhibitor concentrations for which we could follow the reaction (1×10^{-7} to 1×10^{-6} M), the direct plot of k_{in} versus spiramycin concentration showed a linear rather than a hyperbolic dependence (not shown).

$$C + A \overset{K_A}{\rightleftharpoons} CA \underset{k_-}{\overset{k_+}{\rightleftarrows}} C^*A \qquad (2)$$

In eq 2, if k_- is taken to be close to zero, we can determine from Figure 4B an apparent second-order rate constant of inactivation of complex C by spiramycin equal to $k_+/K_A = 2.7 \times 10^4$ M^{-1} s^{-1} ($K_A = 3.1 \times 10^{-6}$ M, $k_+ = 5.0$ min^{-1}), which is close to the value (3.3×10^4 M^{-1} s^{-1}) obtained from the analysis of Scheme II.

*Examination of Spiramycin Complex, C*A.* The spiramycin complex was subjected to ultracentrifugation through a 10–30% linear sucrose gradient at 200000g for 3 h. Spectrophotometric analysis and determination of the radioactivity contained in Ac-[^{3}H]Phe-tRNA showed that this complex remains a 70S ribosomal complex, although it is still inactive to puromycin (results not shown).

The spiramycin complex was further studied by chromatography on Sephadex G-200 (Figure 5A). The spiramycin complex C*A was eluted in the same fractions as complex C. Interestingly, the amount of Ac-Phe-tRNA bound to C*A compared to that bound to complex C showed an increase of about 50% (Figure 5A). This is reminiscent of a similar increase in the amount of Ac-Phe-tRNA bound to complex C in the presence of sparsomycin (Theocharis & Coutsogeorgopoulos, 1992). The ability of C*A to react with puromycin was negligible (see the next section), and it remained negligible even after a second run on the column (see Gel Chromatography). These results indicate that spiramycin inactivates complex C, forming the spiramycin complex which remains a 70S ribosomal complex bearing donor Ac-Phe-tRNA. As will be shown in a later section, spiramycin (A) remains bound to C*A.

*Regeneration of Complex C from the Inactive Spiramycin Complex, C*A.* The distinction between irreversible and slowly reversible inhibition is often only one of degree. Thus, when spiramycin complex C*A is freed of the excess spiramycin by filtration on cellulose nitrate disks or by chromatography on G-200, a very slow recovery of activity is actually observed by both methods, with an apparent pseudo-first-order rate constant equal to 5.0×10^{-5} s^{-1}. This corresponds to k_- of eq 2. The value of $k_- = 5.0 \times 10^{-5}$ s^{-1}, in conjunction with the apparent second-order rate constant $k_+/K_A = 2.7 \times 10^4$ M^{-1} s^{-1} or with the apparent association rate constant $k_{assoc} = 2.7 \times 10^4$ M^{-1} s^{-1} (eq 4, see further), leads to the calculation of an apparent overall dissociation

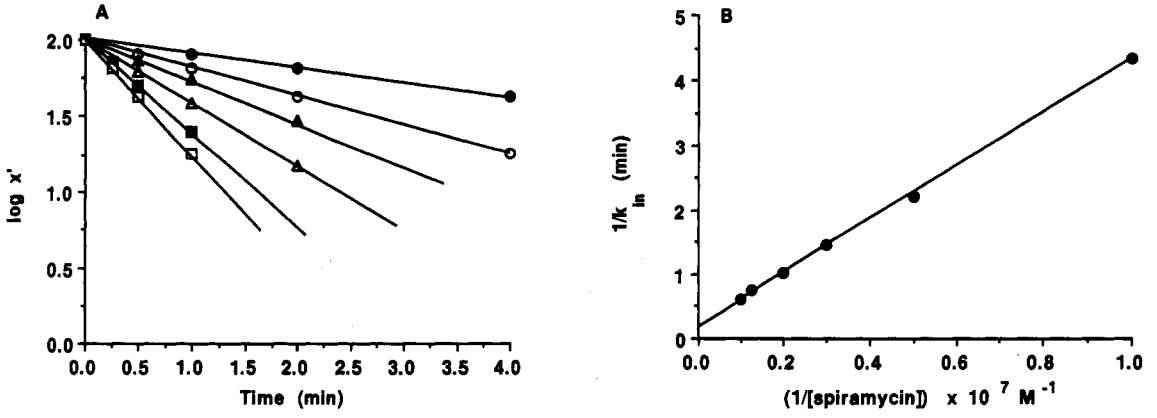


FIGURE 4: (A) First-order time plots for the inactivation of complex C by spiramycin. Complex C in solution (ZW extract) reacted with spiramycin at (●) 0.1 μM, (○) 0.2 μM, (▲) 0.33 μM, (△) 0.5 μM, (■) 0.8 μM, and (□) 1.0 μM for the indicated times of exposure. The percentage (x') of the remaining active complex C is then estimated by titration with puromycin at 2×10^{-3} M for 2 min after the excess spiramycin is removed (for details, see Methods). x' is defined in Theocharis and Coutsogeorgopoulos (1989). (B) Double-reciprocal plot ($1/k_{in}$ versus $1/[A]$) for the inactivation of complex C via the spiramycin reaction. The k_{in} values were taken from A.

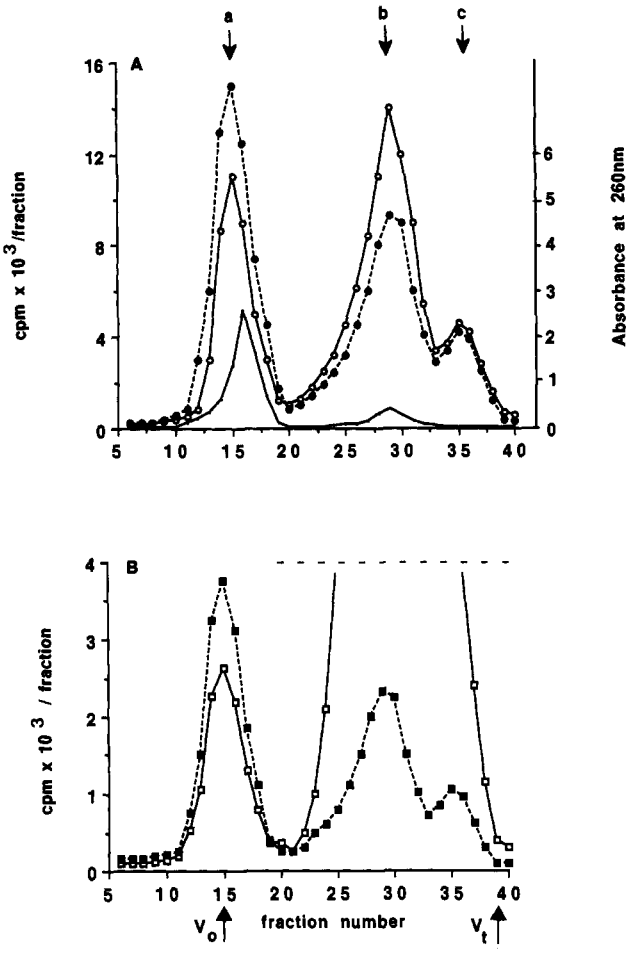


FIGURE 5: Gel chromatography of the binding mixture on a Sephadex G-200 column. (A) Complex C in the binding mixture made (○) in the absence of spiramycin (control) or (●) in the presence of 2×10^{-5}M spiramycin (spiramycin complex C*A). The letters a, b, and c indicate the positions of complex C (or C*A), Ac-[^{3}H]Phe-tRNA, and Ac-[^{3}H]Phe, respectively. V_0 and V_t indicate void volume and total volume of the column, respectively. (B) Double-labeling experiment. The binding mixture was prepared as above using Ac-[^{14}C]Phe-tRNA (500 cpm/pmol) and [^{3}H]dihydrospiramycin (5×10^{-5} M and 350 cpm/pmol) in place of spiramycin: (■) ^{14}C label; (□) ^{3}H label. Absorbance at 260 nm.

constant equal to 1.8 nM for the C*A complex. We have also calculated a dissociation constant from equilibrium dialysis experiments (not shown) in which spiramycin competes with [^{3}H]dihydrospiramycin for binding to the "washed ribosomes" used in the present study. This K_i is equal to 80 nM. Thus, there is a 45-fold difference in the affinity between spiramycin and complex C compared to spiramycin and washed ribosomes. Apparently, the presence of Ac-Phe-tRNA and the ribosomal wash during the binding reaction helps spiramycin form a tighter complex.

Studies with Dihydrospiramycin. The aforementioned studies do not distinguish whether the spiramycin complex carries the antibiotic bound on it or whether spiramycin exerts its effect and subsequently dissociates. In order to clarify the matter, we prepared a derivative of spiramycin in which the aldehyde group was made radioactive by reduction with tritiated borohydride, as described in Methods.

The k_{in} of dihydrospiramycin was determined as in spiramycin through inactivation experiments like those depicted in Figure 4. The $1/k_{in}$ versus 1/[dihydrospiramycin] as well as the k_{in} versus [dihydrospiramycin] plots are linear, giving for a two-step reaction $K_A = 1.6 \times 10^{-5}$ M, $k_+ = 1.8$ min^{-1}, and $k_+/K_A = 2 \times 10^3$ M^{-1} s^{-1}. Comparison of the k_+/K_A ratio of dihydrospiramycin to that of spiramycin (2.7×10^4 M^{-1} s^{-1}) shows a 15-fold difference in potency. Yet, dihydrospiramycin reacts with complex C to produce a complex which, like the spiramycin complex, is inactive toward puromycin. In addition, after chromatography on Sephadex G-200, this complex remains as inactive as it was prior to chromatography. This suggests that dihydrospiramycin also inhibits Ac-Phe-puromycin formation rather irreversibly.

Using tritiated dihydrospiramycin, we showed that, after chromatography on Sephadex G-200, dihydrospiramycin remains bound on complex C (Figure 5B). From the peaks of radioactivity coeluted at V_0 (Figure 5B), it can be calculated that the picomoles of Ac-[^{14}C]Phe-tRNA bound to complex C (30 pmol) are equal to the pmoles of [^{3}H]dihydrospiramycin. The same procedure was repeated using washed ribosomes but omitting Ac-Phe-tRNA and the ribosomal wash from the binding mixture. In this case, 25 pmol instead of 30 pmol of [^{3}H]dihydrospiramycin was bound. When, under these conditions, the ribosomes were doubled, the amount of bound [^{3}H]dihydrospiramycin was increased from 25 to 45 pmol, indicating that the radioactive ligand is used in excess whereas the ribosomes are limiting. From Figure 5B the stoichiometry between dihydrospiramycin and complex C is calculated to be approximately 1:1. Similar behavior for spiramycin itself can be assumed. We conclude that the spiramycin complex C*A is a tight complex and carries spiramycin bound on it.

Reaction of Spiramycin with Complex C in the Presence of Other Inhibitors of Peptidyltransferase. Spiramycin inhibits peptide bond formation in various ribosomal systems [reviewed in Vazquez (1979)]. It was of interest to examine the reaction of complex C with spiramycin in the presence of other known inhibitors of peptidyltransferase, such as the antibiotics chloramphenicol, lincomycin, and sparsomycin. These antibiotics interact with complex C in a two-step reaction as presented in eq 3, where I is chloramphenicol, lincomycin, or sparsomycin.

$$C + I \underset{}{\overset{K_i}{\rightleftharpoons}} CI \underset{k_7}{\overset{k_6}{\rightleftharpoons}} C^*I \qquad (3)$$

(*1*) *Chloramphenicol or Lincomycin.* In this case, complex C*I does not survive filtration through cellulose nitrate disks, and it is possible to examine the inactivation of complex C by spiramycin (spiramycin reaction), i.e., by coupling the reactions of eqs 2 and 3, and to remove the excess spiramycin and chloramphenicol or lincomycin before following the inactivation process. Thus, the inactivation experiments were repeated with the following differences: (a) in place of spiramycin alone, a mixture of lincomycin with spiramycin or a mixture of chloramphenicol with spiramycin was used, and (b) complex C reacted while adsorbed on cellulose nitrate disks. As shown in Figure 6, when a mixture of spiramycin with chloramphenicol or lincomycin was used in place of spiramycin alone, a decrease in the apparent rate constant of inactivation (k_{in}') occurred. When preincubation of complex C with either lincomycin or chloramphenicol precedes the addition of spiramycin, a further decrease in the k_{in} occurs (Figure 6). This effect (the preincubation effect) supports our previous proposal that chloramphenicol and lincomycin react with complex C as slow-binding inhibitors (Theocharis et al., 1992; Kallia-Raftopoulos et al., 1992).

(*2*) *Sparsomycin.* Unlike the corresponding chloramphenicol or lincomycin complexes, the sparsomycin complex C*I can be isolated on cellulose nitrate disks as a mixture with complex C, permitting the determination of k_7 of eq 3 (I =

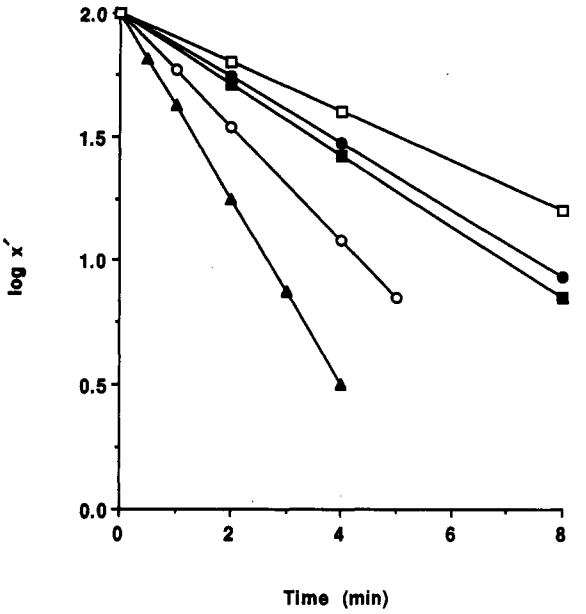


FIGURE 6: First-order time plots for the inactivation of the disk-adsorbed complex C by spiramycin when (▲) spiramycin (5×10^{-6} M) alone is present or (○) spiramycin (5×10^{-6} M) and lincomycin (1×10^{-5} M) or (■) spiramycin (5×10^{-6} M) and chloramphenicol (5×10^{-6} M) are concomitantly present. Complex C is preincubated with (●) lincomycin (1×10^{-5} M) or (□) chloramphenicol (5×10^{-6} M), and then spiramycin (5×10^{-6} M) is added (stage of preincubation). The percentage (x') of the remaining active complex C is determined by titration with puromycin (2 mM, 2 min) after the cellulose nitrate disk is washed to remove the excess antibiotics.

sparsomycin). Such an attempt has been made, but k_7 was not determined unequivocally because of the ensuing kinetic complexity when the puromycin reaction (eq 1) is used to quantitate complex C in a mixture with C*I (Theocharis & Coutsogeorgopoulos, 1992). For this reason, we have used the spiramycin reaction (eq 2) in place of the puromycin reaction. The mixture of complex C and the sparsomycin complex C*I, isolated on cellulose nitrate disks, was exposed to spiramycin at various time intervals. The spiramycin reaction shows two phases giving an early and a late slope (Figure 7A). The value of the late slope depends on the concentration of spiramycin when this is below 1×10^{-5} M. The early slope represents the reaction of spiramycin mostly with preexisting free complex C; the late slope represents the reaction of spiramycin with complex C regenerated from the sparsomycin complex C*I via the k_7 step of eq 3. From the late slope we calculate $k_{late} = 2.2 \times 10^{-3}$ s^{-1}. This finding was double-checked with an independent method as shown in Figure 7B. The mixture of complex C and the sparsomycin complex C*I, isolated on cellulose nitrate disks, was first exposed to reaction buffer at 25 °C for the indicated time intervals. At the end of each exposure, the regenerated complex C was measured by reaction with puromycin (2 mM, 2 min). This method gave a pseudo-first-order rate constant equal to 1.8×10^{-3} s^{-1} (Figure 7B). Since this value is very close to k_{late}, we conclude that the conversion of the sparsomycin complex C*I to the spiramycin complex C*A is achieved via the conversion of C*I to free C through the k_7 step of eq 3 and that $k_7 = (2.0 \pm 0.2) \times 10^{-3}$ s^{-1}.

Reaction of Hydroxylamine with Donor Ac-Phe-tRNA. Hydroxylamine is a reagent which reacts with activated esters to give hydroxamic acids (Eigner & Loftfield, 1981). As shown in Figure 8 (lower line), aqueous hydroxylamine (0.4 M) reacts with Ac-Phe-tRNA which is not bound to ribosomes in a reaction that obeys pseudo-first-order kinetics. Hydroxylamine reacts at a lower rate (Figure 8, middle line) with Ac-Phe-tRNA that is bound to complex C in a puromycin-reactive state. The bound Ac-Phe-tRNA appears to be protected against hydroxylamine. Moreover, when complex C was preincubated with sparsomycin or blasticidin S and then exposed to hydroxylamine, in the continuous presence of sparsomycin or blasticidin S, additional protection was observed compared to that observed when Ac-Phe-tRNA is bound to complex C and has not been exposed to sparsomycin or blasticidin S (Figure 8, upper line). In contrast, spiramycin, chloramphenicol, or lincomycin, under identical conditions, offered no extra protection to Ac-Phe-tRNA (Figure 8, middle line). The second-order rate constant of the reaction of hydroxylamine with Ac-Phe-tRNA bound to complex C was calculated to be equal to 1.3×10^{-3} M^{-1} s^{-1} from the plot of k_{obs} versus [hydroxylamine] (Figure 8, inset). Considering that at 0.4 M hydroxylamine reacts with a pseudo-first-order rate constant of $(1.3 \times 10^{-3})0.4 = 5.2 \times 10^{-4}$ s^{-1} and that k_- in eq 2 is 5.0×10^{-5} s^{-1}, we conclude that hydroxylamine reacts directly with the spiramycin complex C*A without depending on the regeneration of C from C*A. On the other hand, hydroxylamine essentially does not react with the donor of the sparsomycin complex C*I since the pseudo-first-order rate constant is very low (4.8×10^{-5} s^{-1} at 0.4 M hydroxylamine).

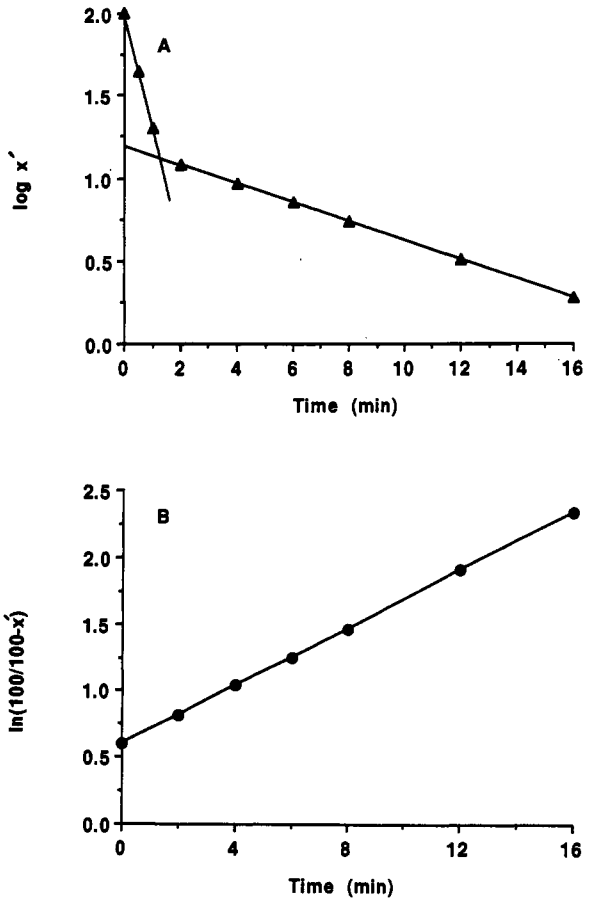


FIGURE 7: Determination of the pseudo-first-order rate constant for the regeneration of complex C from the sparsomycin complex C*I: (A) by the use of the spiramycin reaction at 1×10^{-5} M spiramycin; (B) after exposure to reaction buffer for several time intervals and reaction with 2 mM puromycin for 2 min. More details are given in the text.

DISCUSSION

In the present study, we examined the inhibition of peptide bond formation by one of the macrolides, spiramycin, in a

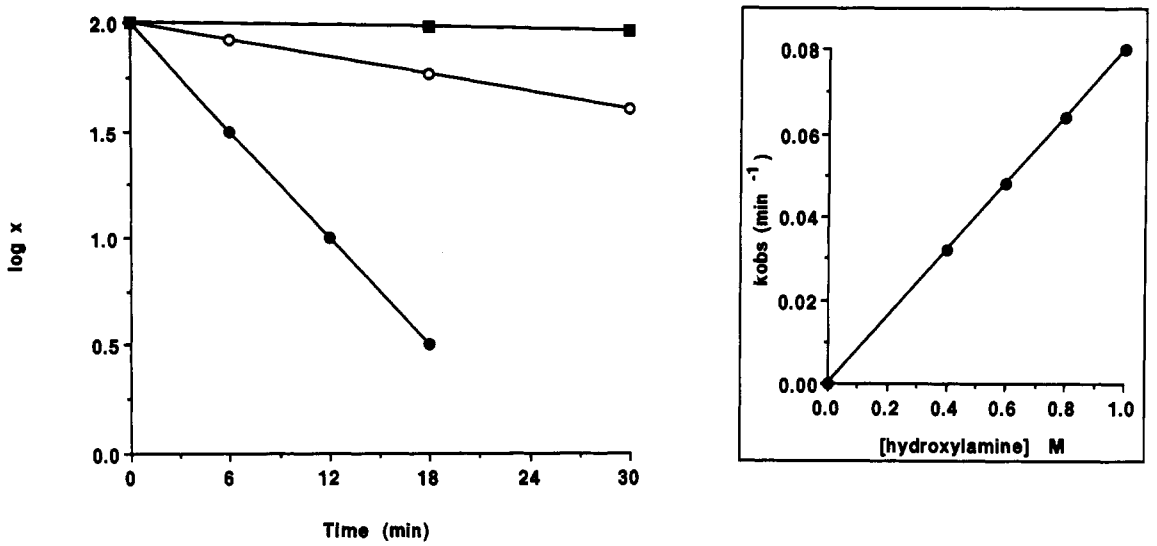


FIGURE 8: First-order time plots for the reaction of hydroxylamine with (●) free Ac-Phe-tRNA, (○) Ac-Phe-tRNA bound to complex C, to the spiramycin complex C*A, or to the putative lincomycin or chloramphenicol complexes C*I (eq 3), and (■) Ac-Phe-tRNA bound to sparsomycin or blasticidin S complexes. The percentage (x) of Ac-Phe-tRNA that did not react with hydroxylamine was calculated as described in Methods. Inset: Pseudo-first-order rate constant k_{obs} for hydroxylaminolysis of Ac-Phe-tRNA bound to complex C as a function of hydroxylamine concentration. k_{obs} at each concentration of hydroxylamine was calculated from plots analogous to that given in Figure 8 (middle line).

model reaction in which peptidyl-tRNA was replaced by Ac-Phe-tRNA. Spiramycin interacted with complex C with an apparent association rate constant on the order of 10^4 M^{-1} s^{-1}. This value is much lower than the upper limit of 10^6 M^{-1} s^{-1} set for the characterization of a molecule as a slow-binding inhibitor (Morrison & Walsh, 1988). On the other hand, spiramycin exhibited a very small rate constant for the regeneration of complex C from complex C*A ($k_- = 5.0\times10^{-5}$ s^{-1}). These facts allow us to characterize spiramycin in the present system as a slow-binding, slowly reversible inhibitor of peptide bond formation.

Spiramycin exhibited a distinctive pattern of inhibition kinetics (Figure 1). In this case, we have difficulty deducing the formation of the conventional encounter complex (CI) between complex C and inhibitor (I) via competitive kinetic plots as we have done successfully in the past for other inhibitors of protein synthesis. Kinetic analysis of the inhibition of peptide bond formation by spiramycin, i.e., when the puromycin and spiramycin reactions are run in parallel, supports the one-step mechanism. On the other hand, the results obtained when the spiramycin reaction was evaluated alone are compatible with both the one- and two-step mechanisms. Thus, the most plausible explanation is that we are dealing with a variation of Scheme I in which the initial encounter complex CA is kinetically insignificant and C*A is detected as the product derived from C and A (eq 4).

$$C + A \underset{k_{dissoc}}{\overset{k_{assoc}}{\rightleftarrows}} C^*A \qquad (4)$$

In the reaction in eq 4, the K_A corresponding to the equilibrium C + A ⇌ CA is so large that CA can be omitted. Similar cases have been described by Kurz and Frieden (1983) and by Morrison and Walsh (1988). The low value of the apparent association rate constant ($k_{assoc} = 2.7\times10^4$ M^{-1} s^{-1}) is compatible with the contention that a conformational change of complex C leads to the formation of a tight complex C*A. We propose this conformational change in analogy to suggestions made for enzymes encountering transition-state analog inhibitors such as (a) the system of adenosine deaminase and 1,6-dihydro-6-(hydroxymethyl)purine riboside with a rather low k_{assoc} (Frieden et al., 1980; Kurz et al., 1987, 1992) or (b) ground-state analog inhibitors which act with the two-step mechanism such as adenosine deaminase and 9-(*erythro*-2-hydroxy-3-nonyl)adenine (Frieden et al., 1980). In the case of spiramycin and complex C, it is conceivable that the fitting difficulties during the encounter result in conformational changes of complex CA, so that the antibiotic becomes bound more strongly.

Erion and Walsh (1987) have reported a similar case in the inhibition of 1-aminocyclopropanecarboxylate deaminase by 1-aminocyclopropane phosphonate. Their kinetic analysis was consistent with the one-step mechanism, albeit with a very low association rate constant (100 M^{-1} s^{-1}). Their probing of the two-step mechanism (i.e., the model of eq 2) on the same results led them to propose that k_+ is sufficiently large so as to hinder detection of the initial weakly associated enzyme–inhibitor intermediate.

The isomerization of CA ⇌ C*A that we propose here has been adopted in a similar model related to the action of lincomycin in the same system (Kallia-Raftopoulos et al., 1992).

In the case of the inactivation of enzymes by an irreversible inhibitor, the type of inhibition may be determined by studying the effect of substrate concentration on the apparent rate constant of inactivation (Tian & Tsou, 1982; Liu & Tsou, 1986; Teruel et al., 1987). In our case, however, complex C is inactivated concomitantly by spiramycin and puromycin. We should therefore use criteria other than those used for the inactivation of enzymes by irreversible inhibitors in order to decide whether there is competition between spiramycin and puromycin for complex C. Thus, for the more plausible one-step mechanism of Scheme II, the competition may be deduced from the linearity of the F versus [A] plot (Figure 2A) in agreement with eq A.2 of the Appendix and from the linearity of the $1/[P_\infty]$ versus $1/[S]$ plot (Figure 3B) in agreement with eq A.9 of the Appendix (after substituting G from eq A.6 and F from eq A.2).

The very low rate constant of regeneration of C from C*A (5.0×10^{-5} s^{-1}) explains why C*A is a tight complex and retains spiramycin following physical separation methods. It can also explain why the sparsomycin complex C*I is converted

quantitatively to C*A by spiramycin in a two-stage process; in contrast, C*A is not convertible to C*I because k_- of eq 2 is much smaller than k_7 of eq 3. In fact, the spiramycin complex is the tightest complex between complex C and any of the inhibitors of peptidyltransferase that we have examined so far, i.e., chloramphenicol, blasticidin S, lincomycin, and sparsomycin. Some of these antibiotics render the bound Ac-Phe-tRNA differentially susceptible to attack by hydroxylamine (Figure 8). While in the spiramycin complex C*A Ac-Phe-tRNA reacts with hydroxylamine uninfluenced by the bound spiramycin, in the sparsomycin complex C*I the donor appears to be protected [present experiments and older evidence supplied by Jimenez et al. (1970)]. It is now of interest to note that blasticidin S behaves like sparsomycin, whereas chloramphenicol and lincomycin behave like spiramycin offering no protection to the bound donor. Apparently, the protection against attack by hydroxylamine is not related to the tightness of the complex between C and the inhibitor.

The spiramycin reaction can supplement the puromycin reaction in the investigation of certain aspects of ribosomal peptide bond formation such as the following.

(1) Detection of a strong preincubation effect in the case of chloramphenicol and lincomycin (Figure 6); this effect, when observed in studies on inhibition of peptide bond formation, constitutes evidence for slow-onset inhibition by chloramphenicol or lincomycin. However, when the puromycin reaction was used to determine product formation, it was difficult to detect a preincubation effect with chloramphenicol (Drainas et al., 1987) or with lincomycin (Kallia-Raftopoulos et al., 1992). The large difference between $k_3/K_S = 10^2\ M^{-1}\ s^{-1}$ (eq 1) for the puromycin reaction and $k_{assoc} = 3.3 \times 10^4\ M^{-1}\ s^{-1}$ (eq 4) for the spiramycin reaction may be one of the factors that contributes to the easier detection of the preincubation effect in the interaction between complex C and chloramphenicol or lincomycin.

(2) Determination of k_7 of eq 3 when I is sparsomycin. In a previous study (Theocharis & Coutsogeorgopoulos, 1992), it was difficult to determine k_7 because puromycin enters into further reaction with C*I. In the present study, where the spiramycin reaction was used, we were able to determine $k_7 = 2.0 \times 10^{-3}\ s^{-1}$. Thus, we can now proceed (a) to calculate the apparent association rate constant (Schloss, 1988) for sparsomycin and complex C as equal to $k_7/K_i' = 1 \times 10^5\ M^{-1}\ s^{-1}$, where $K_i' = K_i(k_7/k_6 + k_7)$ and (b) to explain the preferential conversion of C*I to C*A by spiramycin (Figure 7A), which is due to the large difference between k_7 of eq 3 and k_- of eq 2. The apparent association rate constant of sparsomycin ($1 \times 10^5\ M^{-1}\ s^{-1}$) can now be compared to those of spiramycin ($3 \times 10^4\ M^{-1}\ s^{-1}$) and lincomycin [$1.1 \times 10^4\ M^{-1}\ s^{-1}$; calculated from Kallia-Raftopoulos et al. (1992)]. Information on such constants is scant in the field of inhibitors of peptide bond formation. In fact, association rate constants have been previously determined only for free ribosomes and antibiotics, such as spiramycin, lincomycin, or erythromycin, and were found to be equal to 2.0×10^4, 4.5×10^3, and $3.2 \times 10^5\ M^{-1}\ s^{-1}$, respectively (DiGiambattista et al., 1987). The low values of our association rate constants lend support to the notion that we are dealing with slow-binding inhibitors and provide insight into the rates of possible conformational changes occurring during protein synthesis.

APPENDIX

Complex C is inactivated through two parallel, irreversible reactions, the puromycin reaction and the spiramycin reaction, which take place according to either Scheme I or Scheme II. The spiramycin reaction occurs either (i) through rapid formation of an intermediate complex, CA (two-step mechanism, Scheme I), or (ii) without formation of an intermediate complex (one-step mechanism, Scheme II).

For both schemes, the differential of non-inactivated complex C (C_T) is given by the equation:

$$-\frac{d[C_T]}{dt} = F[C_T] \tag{A.1}$$

where, for the one-step mechanism, $[C_T] = [C] + [CS]$ and

$$F = \frac{k_3[S] + kK_s[A]}{K_s + [S]} \tag{A.2}$$

whereas, for the two-step mechanism, $[C_T] = [C] + [CS] + [CA]$ and

$$F = \frac{k_3[S]}{(1 + [A]/K_A) + [S]} + \frac{k_+[A]}{(1 + [S]/K_s) + [A]} \tag{A.3}$$

By integrating eq A.1, we obtain the values of non-inactivated complex C, C_T, at any time t according to

$$[C_T] = [C_0]e^{-Ft} \tag{A.4}$$

where F is the apparent rate constant of the inactivation of complex C. The rate of product formation is given by $d[P]/dt = k_3[CS]$; if we substitute [CS] with its equivalent as a function of $[C_T]$, we obtain

$$\frac{d[P]}{dt} = Ge^{-Ft} \tag{A.5}$$

where, for the one-step mechanism, the preexponential factor G is given by

$$G = \frac{k_3[S][C_0]}{K_s + [S]} \tag{A.6}$$

whereas, for the two-step mechanism, it is given by

$$G = \frac{k_3[S][C_0]}{K_s(1 + [A]/K_A) + [S]} \tag{A.7}$$

By integrating eq A.5, we obtain

$$[P] = \frac{G}{F}(1 - e^{-Ft}) \tag{A.8}$$

The concentration of product formed when t tends to infinity is

$$[P_\infty] = \frac{G}{F} \tag{A.9}$$

From eqs A.8 and A.9 we obtain

$$\ln (P_\infty - P) = \ln (P_\infty) - Ft \tag{A.10}$$

From the plot of $\ln (P_\infty - P)$ versus t, one can experimentally obtain the apparent rate constant F for the inactivation of complex C.

By plotting F versus [A], G versus [A], and $1/P_\infty$ versus [A] (see Results), we can easily distinguish between Schemes I and II.

REFERENCES

Cooperman, B. S. (1987) *Pharmacol. Ther. 34*, 271–302.

Coutsogeorgopoulos, C., Miller, J. T., & Hann, D. M. (1975) *Nucleic Acids Res. 2*, 1053–1072.

Cundliffe, E. (1987) *Biochimie 69*, 863–869.

DiGiambattista, M., Engelborghs, Y., Nyssen, E., & Cocito, C. (1987) *J. Biol. Chem. 262*, 8591–8597.
DiGiambattista, M., Nyssen, E., Pecher, A., & Cocito, C. (1990) *Biochemistry 29*, 9203–9211.
Drainas, D., Kalpaxis, D. L., & Coutsogeorgopoulos, C. (1987) *Eur. J. Biochem. 164*, 53–58.
Eigner, E. A., & Loftfield, R. B. (1981) in *Selected Methods in Enzymology Series* (Moldave, K., Ed.) pp 256–274, Academic Press, New York.
Erion, M. D., & Walsh, C. T. (1987) *Biochemistry 26*, 3417–3425.
Frieden, C., Kurz, L. C., & Gilbert, H. R. (1980) *Biochemistry 19*, 5303–5309.
Hall, C. C., Johnson, D., & Cooperman, B. S. (1988) *Biochemistry 27*, 3983–3990.
Jimenez, A., Monro, R. E., & Vazquez, D. (1970) *FEBS Lett. 7*, 109–111.
Kallia-Raftopoulos, S., Kalpaxis, D. L., & Coutsogeorgopoulos, C. (1992) *Arch. Biochem. Biophys. 298*, 332–339.
Kurz, L. C., & Frieden, C. (1983) *Biochemistry 22*, 382–389.
Kurz, L. C., Weitkamp, E., & Frieden, C. (1987) *Biochemistry 26*, 3027–3032.
Kurz, L. C., Moix, L., Riley, M. C., & Frieden, C. (1992) *Biochemistry 31*, 39–48.
Leytus, S. P., Toledo, D. L., & Mangel, W. F. (1984) *Biochim. Biophys. Acta 788*, 74–86.
Liu, W., & Tsou, C. L. (1986) *Biochim. Biophys. Acta 870*, 185–190.
Marconi, R. T., Lodmell, J. S., & Hill, W. E. (1990) *J. Biol. Chem. 265*, 7894–7899.
Moazed, D., & Noller, H. F. (1989) *Nature 342*, 142–148.
Morrison, J. F., & Walsh, C. T. (1988) *Adv. Enzymol. Relat. Areas Mol. Biol. 61*, 201–301.
Odom, O. W., & Hardesty, B. (1987) *Biochimie 69*, 925–938.
Odom, O. W., & Hardesty, B. (1992) *J. Biol. Chem. 267*, 19117–19122.
Odom, O. W., Picking, W. D., Tsalkova, T., & Hardesty, B. (1991) *Eur. J. Biochem. 198*, 713–722.
Pestka, S. (1974) *Antimicrob. Agents Chemother. 6*, 474–478.
Schloss, J. V. (1988) *Acc. Chem. Res. 21*, 348–353.
Synetos, D., & Coutsogeorgopoulos, C. (1987) *Biochim. Biophys. Acta 923*, 275–285.
Synetos, D., & Coutsogeorgopoulos, C. (1989) *Eur. J. Biochem. 184*, 47–52.
Tejedor, F., & Ballesta, J. P. G. (1985) *Biochemistry 24*, 467–472.
Tejedor, F., & Ballesta, J. P. G. (1986) *Biochemistry 25*, 7725–7731.
Teruel, J. A., Tudela, J., Belda, F. F., Carmona, F. G., Fernandez, J. C. G., & Canovas, F. G. (1987) *Biochim. Biophys. Acta 911*, 256–260.
Theocharis, D. A., & Coutsogeorgopoulos, C. (1989) *Anal. Biochem.* 176, 278–283.
Theocharis, D. A., & Coutsogeorgopoulos, C. (1992) *Biochemistry 31*, 5861–5868.
Theocharis, D. A., Synetos, D., Kalpaxis, D. L., Drainas, D., & Coutsogeorgopoulos, C. (1992) *Arch. Biochem. Biophys. 292*, 266–272.
Tian, W. X., & Tsou, C. L. (1982) *Biochemistry 21*, 1028–1032.
Vazquez, D. (1967) *Life Sci. 6*, 381–386.
Vazquez, D. (1979) *Mol. Biol. Biochem. Biophys. 30*, 1–312.
Williams, J. W., & Morrison, J. F. (1979) *Methods Enzymol. 63*, 437–467.