Interaction between the Erythromycin and Chloramphenicol Binding Sites on the *Escherichia coli* Ribosome†

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ABSTRACT: The effects of chloramphenicol on the binding kinetics of a fluorescein isothiocyanate derivative of 9(*S*)-erythromycylamine with 70S and 50S ribosomes have been studied by direct fluorimetric measurements. While chloramphenicol had little effect on the second-order 70S binding rate of the erythromycin analogue, it substantially reduced the dissociation rate of the fluorescent antibiotic–70S ribosome complex. This could be explained by simultaneous binding of both antibiotics to the 70S ribosome. The kinetic results suggest that chloramphenicol-saturated 70S particles bind the erythromycin analogue four times stronger and this was confirmed by direct binding studies. In addition, chloramphenicol causes a twofold increase in the intrinsic fluorescence of the 70S-bound analogue. This increase in fluorescence was used to study the kinetics of chloramphenicol binding to 70S ribosomes containing the fluorescent derivative. The fluorescence change followed first-order kinetics, suggesting that chloramphenicol induces a conformational change in the 70S particle. This could explain both its effect on erythromycin binding and on the fluorescence of bound analogue. Less detailed results with the 50S particle indicate a qualitatively similar picture of erythromycin–chloramphenicol interactions.

Numerous antibiotics have been shown to bind to bacterial ribosomes and inhibit protein synthesis (Pestka, 1971; Davis et al., 1974). To understand the action of these drugs it is necessary to define the steps in protein synthesis they inhibit and also to localize their binding sites. If a more detailed picture of the action of an antibiotic is desired, additional information must be sought about the mechanism of its interaction with the ribosome. The most direct approach for this is equilibrium binding measurements (Pestka, 1974b). A number of different experimental techniques are available for this purpose but most have serious limitations. Even when these are circumvented many complex mechanisms cannot be distinguished by equilibrium studies alone. Kinetic studies can provide a more detailed picture of binding reactions particularly when used in concert with equilibrium results. Such studies have been relatively rare with ribosomes because few antibiotics have properties allowing convenient direct monitoring of the binding reactions.

A fluorescent derivative of the macrolide antibiotic erythromycin has been synthesized (Vince et al., 1976). Previous studies have shown that this derivative F-Ery[1] competes with erythromycin for binding to the 50S subunit. It is reasonable to assume that F-Ery and Ery have the same binding site on the ribosome. The fluorescence of F-Ery is altered when it is bound to the ribosome (Langlois et al., 1976). Thus, fluorescence measurements can monitor the kinetics and equilibria between this erythromycin derivative and ribosomes. The high sensitivity of fluorescence techniques enables measurements at very low concentrations, minimizing the problem of secondary binding and nonspecific interactions. Also, the concentrations of the free and bound antibiotic in a sample can be directly determined without procedures which might perturb the binding. In this paper, we report the results of fluorescence experiments on the binding of F-Ery to 50S and 70S ribosomes. Particular attention will be focused on how these interactions are altered by the presence of another antibiotic, chloramphenicol.

Experimental Section

Samples. Salt-washed 70S ribosomes and 50S subunits were prepared from early log *Escherichia coli* MRE600 cells as described previously (Langlois et al., 1976). The fluorescein isothiocyanate derivative of 9(*S*)-erythromycylamine, F-Ery, was prepared from fluorescein isothiocyanate and 9(*S*)-erythromycylamine according to the procedure of Vince et al. (1976).

Fluorescence Measurements. The fluorescence measurements were all performed at 25 °C in the following buffer: 10 mM Tris-HCl (pH 7.4)–10 mM $MgCl_2$–30 mM NH_4Cl–100 mM KCl–5 mM β-mercaptoethanol. A Schoeffel RRS-1000 spectrofluorimeter interfaced with a Tektronics E31 programmable calculator was used for fluorescence measurements. For kinetic studies the instrument was programmed to automatically collect data at either 5-s intervals (70S experiments) or 10-s intervals (50S experiments). The excitation and emission wavelengths were 490 and 520 nm, respectively. Throughout the study, light scattering was less than 1% of the intensity of fluorescence emission.

Analysis of Kinetic Data. Kinetic data corresponding to a unimolecular process were analyzed by the following first-order rate equation:

$$\ln\left[\frac{I(t) - I_\infty}{I_0 - I_\infty}\right] = Y = -kt \qquad (1)$$

where I_0, $I(t)$, and I_∞ are the fluorescence values of the sample at $t = 0$, $t = t$, and $t = \infty$, respectively. The first-order rate constant was determined from the slope of a least-squares fit of Y vs. t. Kinetic data corresponding to a bimolecular process were analyzed by the following second-order rate equations:

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[1] Abbreviations used are: Ery, erythromycin; F-Ery, the fluorescein isothiocyanate derivative of 9(*S*)-erythromycylamine [9(*S*)-erythromycylamino-[5-(*N*-carbothioamino)-2-(3-hydroxy-6-oxoxanthen-9-yl)]-benzoic acid]; Tris, tris(hydroxymethyl)aminomethane; in equations and figures CM is used for chloramphenicol.

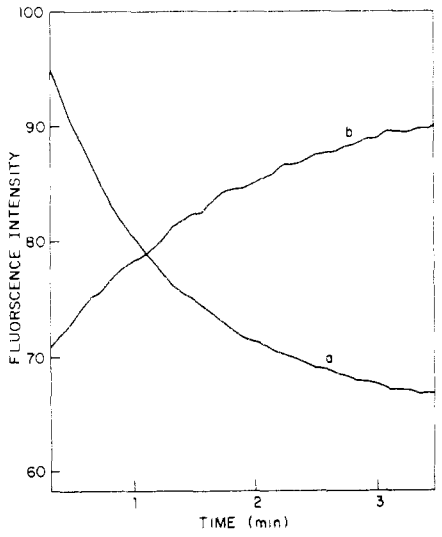

FIGURE 1: Fluorescence intensity of F-Ery as a function of time: (curve a) 70S ribosomes added at time zero to a solution of F-Ery; (curve b) excess erythromycin (2.5×10^{-5} M) added at time zero to a solution of F-Ery · 70S. Final concentrations are the same as in Table II.

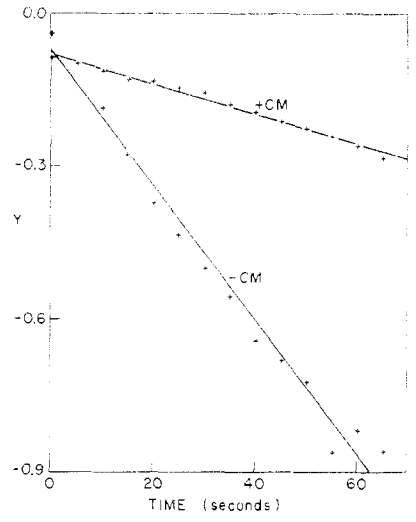

FIGURE 2: First-order exchange kinetics of erythromycin with F-Ery · 70S in the presence and absence of chloramphenicol plotted according to eq 1. The lines are least-squares fits of the data giving k_{-1}(−chloramphenicol) = 0.530 min^{-1} and k_{-1}(+chloramphenicol) = 0.118 min^{-1}.

$$\frac{1}{a-b}\ln\left[\frac{b(a-x)}{a(b-x)}\right] = Y = kt$$
$$x = \left[\frac{I_0 - I(t)}{I_0 - I_\infty}\right]a \quad \text{where} \quad a < b \tag{2}$$

where a is the initial concentration of reactant A; b is the initial concentration of reactant B; x is the concentration of product at time t; and I_0, $I(t)$, and I_∞ are the same as defined for eq 1. For a reversible reaction, these equations are valid only at early times in the reaction. The second-order rate constant was also derived from the slope of a least-squares fit of Y vs. t. In cases where there was uncertainty about the order of a process, the order was verified by determining the rate constant as a function of concentration since true unimolecular and bimolecular rate constants are concentration independent.

Analysis of Equilibrium Binding Data. Equilibrium binding experiments were performed by measuring the fluorescence intensity of F-Ery at equilibrium as a function of the ribosome concentration. The following equation was used to analyze this equilibrium data (Mulvey et al., 1973):

$$\frac{F_F - F}{R} = K_a(F_F - F_B) - K_a(F_F - F) \quad \text{where} \quad R > [\text{F-Ery}] \tag{3}$$

where R is the initial ribosome concentration; F_F is the fluorescence of F-Ery in the absence of ribosomes; F_B is the fluorescence of F-Ery bound to ribosomes; F is the fluorescence of F-Ery solution when the ribosome concentration is R; and K_a is the association constant of F-Ery with the ribosome. This equation has been derived on the assumption that there is one binding site per ribosome which is consistent with previous studies (Pestka, 1974b; Vince et al., 1976). The association constant, K_a, and the fluorescence of bound F-Ery, F_B, were derived from a least-squares fit of $(F_F - F)/R$ as a function of $(F_F - F)$.

Results

Binding of F-Ery to 70S Ribosomes. The macrolide antibiotic erythromycin binds reversibly to one strong binding site on the 50S subunit of *E. coli* ribosomes (Pestka, 1974b). The fluorescein isothiocyanate derivative of 9(*S*)-erythromycylamine (F-Ery) binds to the same site with a weaker binding constant (Vince et al., 1976; Langlois et al., 1976). Since the fluorescence of F-Ery decreased upon binding to 50S subunits and to 70S ribosomes (Langlois et al., 1976), the kinetics of F-Ery binding to ribosomes can be studied directly by monitoring the fluorescence. Figure 1, curve a, shows the fluorescence intensity as a function of time when 70S ribosomes are added to a solution of F-Ery. The changes observed should correspond to the reaction:

$$\text{F-Ery} + 70\text{S} \xrightarrow{k_1} 70\text{S} \cdot \text{F-Ery}$$

To test this mechanism the fluorescence data were treated using eq 2 as described in the Experimental Section. The resulting second-order plot is in excellent agreement with the experimental data. This suggests that F-Ery binding is a biomolecular process. A value for k_1 of 1.34×10^6 M^{-1} min^{-1} is obtained from a least-squares fit of the experimental data to eq 2.

Unlabeled erythromycin displaces F-Ery from the ribosome since it binds to the same site with higher affinity. In the presence of a large excess of erythromycin, the probability of reassociation of a molecule of F-Ery with ribosomes is extremely small. The following scheme illustrates the effect of adding a large excess of erythromycin to a solution of F-Ery and ribosomes at equilibrium. It is assumed that dissociation of the 70S·F-Ery complex is the first step in the exchange reaction:

$$70\text{S} \cdot \text{F-Ery} \xrightarrow{k_{-1}} \text{F-Ery} + 70\text{S}$$

$$70\text{S} + \text{Ery} \rightarrow 70\text{S} \cdot \text{Ery}$$

The only step in this process which affects the fluorescence of F-Ery is its dissociation from the ribosome (k_{-1}). This dissociation leads to an increase in fluorescence as illustrated by the data shown in Figure 1b. If F-Ery·70S dissociation is rate limiting, the exchange kinetics should appear to be a unimolecular process. Thus, the data were analyzed using eq 1 of the Experimental Section. The resulting first-order kinetic plot is shown in Figure 2. It fits the observed data fairly well, supporting the hypothesized reaction scheme. The values of the

TABLE I: Effects of Antibiotics on F-Ery Binding to 70S Ribosomes.[a]

Antibiotic	Concn (M)	k_1 (M^{-1} min^{-1})	k_{-1} (min^{-1})	K_D (M)
None		1.36×10^6	0.563	4.14×10^{-7}
Chloramphenicol	1×10^{-3}	1.27×10^6	0.130	1.02×10^{-7}
PA114B	1×10^{-6}	$\sim 0.14 \times 10^6$	~0.05	$\sim 3 \times 10^{-7}$
Niddamycin	5×10^{-6}	N.B.[b]		

[a] Concentrations used were 6.65×10^{-7} M 70S ribosomes, 1.33×10^{-7} M F-Ery, and 2.5×10^{-5} M Ery. Ribosomes were equilibrated with the test antibiotic at the above concentrations before F-Ery was added. k_1 and k_{-1} correspond to the association and dissociation rate constants, respectively. Data shown for the control and chloramphenicol samples are the average of several determinations. [b] N.B. indicates no fluorescence changes were observed suggesting no binding of F-Ery.

TABLE II: Dissociation Constants for the 70S-F-Ery Complex Determined by Various Methods.

Sample	Kinetics[a] K_D (M)	Equilibrium binding[b] K_D (M)	F_B/F_F	F^c	Filter assay[d] K_D (M)
70S + F-Ery	4.14×10^{-7}	4.11×10^{-7}	0.369	0.62	4.47×10^{-7}
70S·chloramphenicol + F-Ery	1.02×10^{-7}	1.27×10^{-7}	0.664	0.72	

[a] Concentrations same as Table I ± 10^{-3} M chloramphenicol. [b] Concentrations for 70S binding were 1.33×10^{-7} M F-Ery; $3\text{-}13 \times 10^{-7}$ M 70S ribosomes. Concentrations for 70S·chloramphenicol binding were 3.3×10^{-8} M F-Ery; 1×10^{-3} M chloramphenicol, and $1.6\text{-}5 \times 10^{-7}$ M 70S ribosomes. [c] Relative fluorescence at equilibrium of F-Ery in solution in the presence of 70S or 70S·chloramphenicol at the concentrations shown in Table I. [d] Vince et al. (1976).

two rate constants, k_1 and k_{-1}, determined by averaging the results of several experiments, are presented in Table I. An experiment performed with a fourfold dilution of the concentrations of both F-Ery and 70S ribosomes yielded similar rate constants, verifying that k_1 and k_{-1} correspond to bimolecular and unimolecular processes in accordance with the above mechanism.

The dissociation constant of the 70S·F-Ery complex can be derived from the ratio of the rate constants, k_1/k_{-1}. This is shown in Tables I and II.

By monitoring the fluorescence of F-Ery at equilibrium as a function of the ribosome concentration, a second estimate of the dissociation constant can be obtained by using eq 3 as described in the Experimental Section. The resulting modified Scatchard plot is shown in Figure 3. The average value for the dissociation constant determined from two equilibrium experiments is shown in Table II. This table also gives the equilibrium constant determined from a competition experiment between F-Ery and [^{14}C]erythromycin by Vince et al. (1976). It is clear that there is excellent agreement among these three techniques.

The Effects of Other Antibiotics on F-Ery Binding to 70S Ribosomes. The kinetic procedure described above requires only one sample to determine rates and equilibria. It is much simpler than equilibrium binding procedures and potentially yields more information about the interaction of F-Ery with ribosomes. For these reasons, we studied the effects of other antibiotics on F-Ery binding to ribosomes by the kinetic approach. Estimates of the binding affinity of each antibiotic with 70S ribosomes were used to determine the concentrations for testing (Pestka, 1971). Most were used in the range where around 90% of maximum ribosome binding is expected. Of the many antibiotics tested, only three significantly altered the observed kinetics of F-Ery binding to ribosomes. Most antibiotics had no significant effect on the F-Ery binding or exchange rate constants. Table III lists the antibiotics which had no significant effects on the kinetics of F-Ery binding to the ribosome. The rate constants obtained from the three antibiotics which significantly affected F-Ery binding kinetics are shown in Table I. The most interesting result is chloramphenicol which substantially decreases the dissociation rate of F-Ery, as shown in Figure 2, but has little effect on the forward rate. An experiment in the presence of chloramphenicol and fourfold higher concentrations of ribosomes and F-Ery yielded the same rate constants verifying that the binding is still second order while the exchange is still first order.

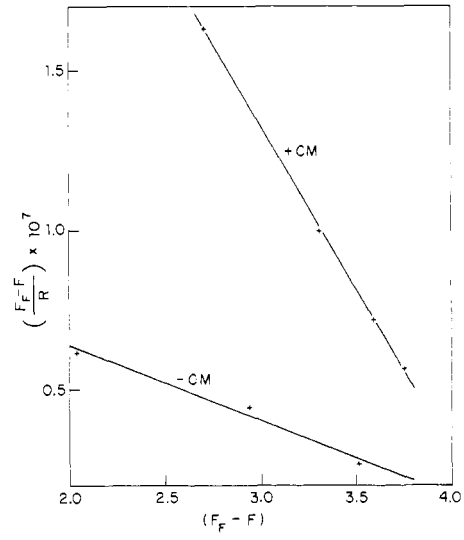

FIGURE 3: Equilibrium binding of F-Ery to 70S ribosomes in the presence and absence of chloramphenicol plotted according to eq 3. The lines are least-squares fits of the data giving K_d(−chloramphenicol) = 4.28×10^{-7} M^{-1} and K_d(+chloramphenicol) = 0.97×10^{-7} M^{-1}. The scales of both the x and the y axes should be multiplied by 0.1 for the −chloramphenicol data. Concentrations were the same as in Table I.

Binding of F-Ery to Ribosomes in the Presence of Chloramphenicol. The results reported in Table I suggest that chloramphenicol enhances the binding affinity of F-Ery to 70S ribosome. To account for the decreased dissociation rate in any simple way it is necessary to postulate the existence of ternary

TABLE III: Antibiotics Which Had No Effect on F-Ery Binding to 70S Ribosomes.[a]

Antibiotic	Concn. tested (M)
Neomycin sulfate	10^{-5}
Fusidic acid	10^{-4}
Amicetin	10^{-4}
Pactamycin	10^{-5}
Streptomycin	10^{-5}
Capreomycin	7 μg/mL
Thiostrepton	10^{-6}
Micrococcin	10^{-6}
Kasugamycin	10^{-4}
X 5108	10^{-5}
Gougerotin	10^{-4}

[a] Conditions used for testing are the same as in Table II. All rate constants obtained in the presence of these antibiotics were within 20% of the control.

TABLE IV: Effect of Chloramphenicol Concentration on Various Rates.[a]

Chloramphenicol concn (M)	k_{-1} (min^{-1})	k_1 (M^{-1} min^{-1})	k_3 (min^{-1})
0	0.512	1.36×10^6	
1.25×10^{-6}	0.446[b]		
5×10^{-6}	0.334[b]		1.1
1×10^{-5}	0.095		1.4
2.5×10^{-5}			1.35
1×10^{-4}	0.144	1.20×10^6	1.4
5×10^{-4}	0.121	1.19×10^6	
1×10^{-3}	0.130	1.21×10^6	
5×10^{-3}	0.138	1.23×10^6	

[a] Rate constant subscripts correspond to steps shown in Figure 5. For [chloramphenicol] = 0, k_{-1} and k_1 correspond, respectively, to k_{-2} and k_2 of Figure 5. [b] First-order kinetic plot was nonlinear suggesting dissociation of F-Ery from both 70S·F-Ery and chloramphenicol·70S·F-Ery. Concentrations used were the same as in Table I.

chloramphenicol·70S·F-Ery complex. This conclusion conflicts with several reports in the literature that chloramphenicol and Ery compete for the same or overlapping sites on the ribosome (see discussion below). For this reason we examined the effect of chloramphenicol on F-Ery binding in greater detail. The association constant for chloramphenicol binding to salt-washed *E. coli* ribosomes has been determined to be 4.3×10^5 M^{-1} (Lessard and Pestka, 1972). At a chloramphenicol concentration of 1.0×10^{-3} M and a ribosome concentration of 6.65×10^{-7} M we calculate that at equilibrium greater than 99% of the ribosomes will contain chloramphenicol in the absence of F-Ery. This would suggest that the F-Ery binding and dissociation rate constants we have observed in the presence of chloramphenicol should correspond to the reactions:

$$\text{CM} \cdot 70\text{S} + \text{F-Ery} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{CM} \cdot 70\text{S} \cdot \text{F-Ery}$$

Equilibrium binding experiments in the presence of 10^{-3} M chloramphenicol were then performed and the data analyzed by eq 3. The resulting modified Scatchard plot is shown in Figure 3. There is substantial agreement between the equilibrium constant determined by this method with that estimated from the kinetic data. The results are shown in Table II. F-Ery appears to bind 2 to 3 times stronger to 70S particles in the presence of chloramphenicol.

F-Ery Binding to 70S Ribosomes as a Function of Chloramphenicol Concentration. The results reported above strongly suggest that the rate constants we observed in the presence of chloramphenicol correspond to direct binding to or dissociation of F-Ery from a chloramphenicol–70S ribosome complex. To further verify this assumption, these rate constants were studied as a function of the chloramphenicol concentration. The bimolecular association constant of F-Ery binding to ribosomes in the presence of chloramphenicol did not change over a chloramphenicol concentration range of 1×10^{-4} to 5×10^{-3} M as shown in Table IV. This strongly suggests that F-Ery is binding to ribosomes containing chloramphenicol since at the highest chloramphenicol concentration used virtually all of the ribosomes contain bound chloramphenicol. This association rate constant is nearly identical with the rate constant in the absence of chloramphenicol; thus from kinetic data alone one cannot be sure that the F-Ery preferentially binds to ribosomes containing chloramphenicol.

The dissociation rate constant of ribosome-bound F-Ery is much slower in the presence of chloramphenicol. The chloramphenicol concentration dependence of this rate constant should therefore give a clearer picture of the effect of chloramphenicol on F-Ery binding. The results of a series of experiments on this dissociation rate are given in Table IV. At very low chloramphenicol concentrations the first-order kinetic plots were not linear. Our results are consistent with F-Ery dissociating from a mixture of 70S ribosomes with and without bound chloramphenicol. This is reasonable since at chloramphenicol concentrations below 10^{-5} M one can expect that not all ribosomes containing F-Ery should also contain bound chloramphenicol. At higher chloramphenicol concentrations a single first-order rate constant is seen for F-Ery exchange. Here the fraction of ribosome lacking chloramphenicol should be too small to contribute significantly to the data so this rate constant presumably represents purely the dissociation of F-Ery from a chloramphenicol·70S·F-Ery ternary complex. This rate constant is fairly insensitive to further increases in chloramphenicol concentration although there is a slight tendency for the rate of F-Ery release to increase somewhat at very high chloramphenicol levels. Since the apparent dissociation rate is somewhat variable over the concentration range of 10^{-5}–5×10^{-3} M, we have used an average value of these results for estimating binding constants from the kinetics.

Effect of Chloramphenicol on the Fluorescence of Bound F-Ery. One surprising result of the fluorimetric binding studies is that the fluorescence of F-Ery bound to chloramphenicol-containing 70S particles is almost twice that of F-Ery bound to chloramphenol-free 70S particles. This is shown in Table II. It may be due to direct interaction between the antibiotics or a conformational change produced by chloramphenicol. The data in Table II are indirect. To confirm it, chloramphenicol was added to a preequilibrated sample of 70S particles and F-Ery. The fluorescence intensity showed a time-dependent increase. The observation that addition of chloramphenicol to 70S·F-Ery causes a fluorescence increase might at first seem to suggest that bound F-Ery is being displaced. This would correspond to the reactions:

$$70\text{S} \cdot \text{F-Ery} \rightleftarrows 70\text{S} + \text{F-Ery}$$

$$70\text{S} + \text{CM} \rightleftarrows 70\text{S} \cdot \text{CM}$$

However, the observed rate of change of fluorescence is an order of magnitude too fast to correspond to dissociation of 70S·F-Ery. The chloramphenicol-induced increase in fluo-

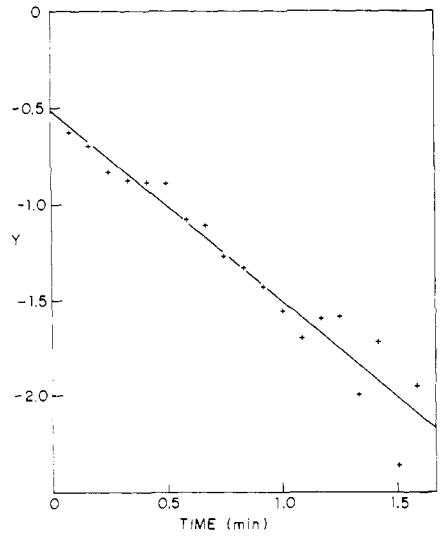

FIGURE 4: First-order kinetic plot of data obtained when chloramphenicol is added to F-Ery · 70S. Concentrations were 6.65×10^{-7} M 70S ribosome, 1.33×10^{-7} M F-Ery, and 5×10^{-6} M chloramphenicol. The line is a least-squares fit of the data giving $k_3 = 1.00$ min^{-1}.

rescence also cannot be explained simply by additional binding of F-Ery in the presence of chloramphenicol since the fluorescence of bound F-Ery is less than free F-Ery in the presence or absence of chloramphenicol. Thus the increase must be a direct effect of the properties of 70S-bound F-Ery.

The simplest possible reaction scheme to explain the chloramphenicol-induced fluorescence increase would be a bimolecular collision:

$$\text{CM} + 70\text{S} \cdot \text{F-Ery} \rightarrow \text{CM} \cdot 70\text{S} \cdot \text{F-Ery}$$

However, when the observed data were fit to eq 2, the bimolecular rate constants derived varied proportionally with varying chloramphenicol concentrations. This suggested that we were in fact observing a unimolecular process, the slow reaction represented by the following scheme:

$$\text{CM} + 70\text{S} \cdot \text{F-Ery} \overset{\text{fast}}{\rightleftarrows} (\text{CM} \cdot 70\text{S} \cdot \text{F-Ery}) \underset{k_{3'}}{\overset{\text{slow}}{\longrightarrow}} \text{CM} \cdot 70\text{S} \cdot \text{F-Ery}$$

This will show simple first-order kinetics if essentially all of the 70S·F-Ery is rapidly converted to (chloramphenicol·70S·F-Ery) and the fluorescence change is associated with the slow step. Therefore, the experimental data were then analyzed by eq 1. A typical first-order plot is shown in Figure 4 and clearly fits the results very well. Values of k_3 are summarized in Table IV. This unimolecular rate constant corresponding to a conformational change of the ternary complex was essentially independent of chloramphenicol concentration although the precision is low because the fluorescence change for this process was small.

One possible kinetic scheme for the interactions of chloramphenicol and F-Ery with *E. coli* 70S ribosomes which is consistent with all of the data at our disposal is shown in Figure 5. Such a cyclic scheme always raises certain ambiguities about reaction pathways. For example, when chloramphenicol is added to preequilibrated 70S·F-Ery, in principle chloramphenicol·70S·F-Ery can be formed either directly (counterclockwise reaction) or after dissociation of 70S·F-Ery (clockwise reaction). However, the first-order rate of this step (k_{-2}) was measured independently and is almost three times

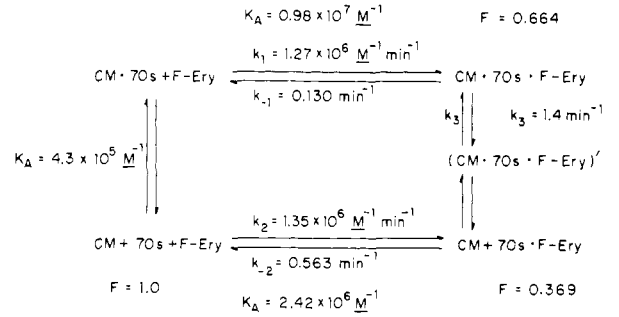

FIGURE 5: Proposed kinetic scheme for the interactions of F-Ery and chloramphenicol with the *E. coli* 70S ribosome. *F* values correspond to the relative fluorescence of F-Ery in each of the pure states.

TABLE V: Summary of Erythromycin Rate Constants with Ribosomes.[a]

Sample	k_1 (M^{-1} min^{-1})	k_{-1} (min^{-1})	K_D (M)
Ery + 70S[b]	1.5×10^7	0.15	1.01×10^{-8}
F-Ery + 70S	1.36×10^6	0.563	4.14×10^{-7}
F-Ery + 70S· chloramphenicol	1.27×10^6	0.130	1.02×10^{-7}
F-Ery + 50S	1.25×10^5	0.138	1.10×10^{-6}
F-Ery + 50S· chloramphenicol	4.59×10^4		

[a] Concentrations for 50S binding used were 2×10^{-6} M 50S ribosome, 1.33×10^{-7} M F-Ery, 2.5×10^{-5} M Ery and $\pm 1 \times 10^{-3}$ M chloramphenicol. Concentrations for 70S samples were the same as in Table I. [b] Vince et al. (1976).

slower than the observed rate of ternary complex formation. Therefore, the bulk of the reaction between chloramphenicol and 70S·F-Ery must proceed directly.

Preliminary Studies on the Interaction of Chloramphenicol and F-Ery with E. coli 50S Subunits. The binding of F-Ery to *E. coli* 50S subunits as measured by this kinetic procedure has been reported previously (Langlois et al., 1976). The rate and equilibrium constants obtained are shown in Table V. These experiments were repeated in the presence of 10^{-3} M chloramphenicol and these results are also shown in Table V. Both the association and dissociation rates were reduced by the presence of chloramphenicol. The dissociation rate became too slow to be measured on this time scale so we were unable to determine an equilibrium constant. This suggests that chloramphenicol also enhances F-Ery binding to 50S particles although this could not be quantitatively measured. The fluorescence change due to F-Ery binding in the presence of chloramphenicol was only ~10% which was too small for our equilibrium binding procedure. Figure 6 shows the fluorescence as a function of time when 50S subunits are added to a solution of F-Ery. After equilibration the addition of chloramphenicol resulted in a large fluorescence increase. This increase was measured at 10^{-3} and 10^{-4} M chloramphenicol. The unimolecular rate constant of 2.27 min^{-1} was found to be independent of the chloramphenicol concentration. Figure 7 also illustrates that the rate of the dissociation of F-Ery from the 50S particle in the presence of chloramphenicol is too slow to be measured.

The results of these preliminary 50S experiments appear to be qualitatively similar to those for the 70S ribosome. Figure 7 summarizes these results according to the same kinetic scheme as postulated for the 70S ribosome. It must be stressed, however, that at present there is insufficient data with the 50S

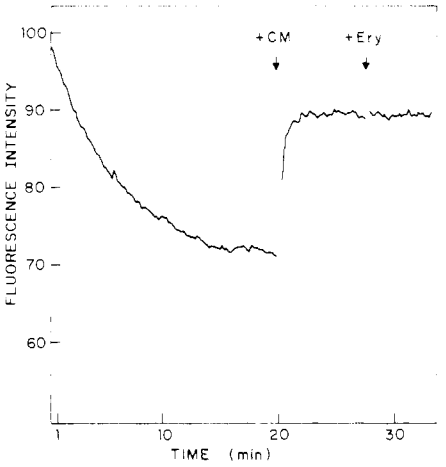

FIGURE 6: Fluorescence intensity of F-Ery as a function of time. 50S particles added at time zero to a solution of F-Ery. Other additions were made as noted. Final concentrations were 2×10^{-6} M 50S, 1.33×10^{-7} M F-Ery, 1×10^{-3} M chloramphenicol, and 2.5×10^{-5} M Ery.

particle to firmly establish this reaction scheme. One interesting difference between the 50S and 70S complexes is apparent. The fluorescence intensity of F-Ery bound to 70S ribosomes is much less than that bound to 50S particles. This indicates that the local environment in the site differs, a result consistent with the different binding strengths of F-Ery to 70S and 50S particles. Chloramphenicol increases the fluorescence of bound F-Ery, both on the 50S and 70S particles. Thus, at least qualitatively, whatever the mechanism of interaction between the two binding sites, it is generally similar in both isolated 50S subunits and intact 70S ribosomes.

Discussion

Erythromycin is a macrolide antibiotic which has a single strong binding site on the 50S subunit of *E. coli* ribosomes and is involved in inhibiting elongation (Pestka, 1971, 1977). The precise mechanism of this inhibition is still unclear. It was suggested that the effects of erythromycin vary depending on the length of the peptide being synthesized (Pestka, 1971, 1977; Mao and Robishaw, 1972; Davis et al., 1974; Otaka and Kaji, 1975). Many studies have evaluated the effect of other antibiotics on erythromycin binding in an attempt to clarify the mechanism of its action. These have often led to conflicting conclusions particularly with respect to the effect of chloramphenicol on erythromycin binding (Fernandez-Muñoz et al., 1971). For these reasons we studied the effects of other antibiotics on the binding of fluorescein erthromycylamine.

Niddamycin is a macrolide antibiotic whose structure is similar to erythromycin. No fluorescence changes were observed when F-Ery was added to ribosomes containing niddamycin suggesting that this antibiotic like most macrolides blocks F-Ery binding. This observation is consistent with competition studies indicating that niddamycin competes with erythromycin for the same site on *E. coli* ribosomes, and that it has an association constant slightly larger than that of Ery (Pestka et al., 1974). Only two of the nonmacrolide antibiotics tested had a significant effect on F-Ery binding kinetics. PA114B and vernamycin Bα are antibiotics of the streptogramin B group. It has been reported that erythromycin and other macrolides inhibit binding of vernamycin Bα to *E. coli* ribosomes and that vernamycin Bα binds to 50S subunits (Ennis, 1974). Our kinetic results indicate that at 10^{-6} M PA114B has a large effect on F-Ery binding to ribosomes. This indicates that this antibiotic has a strong affinity for *E. coli*

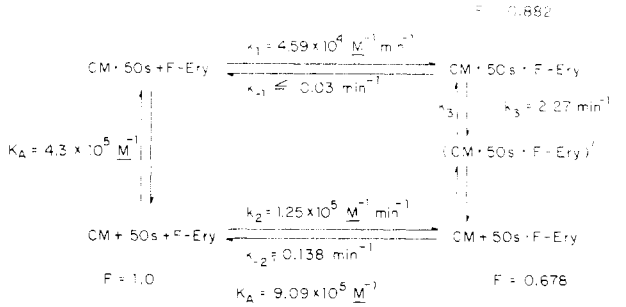

FIGURE 7: Proposed kinetic scheme for the interactions of F-Ery and chloramphenicol with the *E. coli* 50S subunit. *F* values correspond to the relative fluorescence of a solution of F-Ery in the presence of the other components.

ribosomes and is consistent with its binding to 50S subunits. The rate constants obtained for F-Ery binding in the presence of PA114B were too small, however, to determine the overall effect of this antibiotic on F-Ery binding.

Chloramphenicol is known to bind to the 50S subunit of *E. coli* ribosomes (for a review see Pestka, 1971). The association constant of chloramphenicol with salt-washed *E. coli* 70S ribosome is 4.3×10^5 M^{-1} (Lessard and Pestka, 1972) which is much smaller than the association constant of erythromycin with ribosomes. We have observed that chloramphenicol has very little effect on the association rate of F-Ery with 70S ribosomes, but that it greatly reduces the dissociation rate of F-Ery. This result indicates that chloramphenicol may actually enhance F-Ery binding to ribosomes. Equilibrium fluorescence studies with F-Ery in the presence of chloramphenicol are consistent with this.

One possible kinetic scheme which is consistent with all our chloramphenicol data is presented in Figure 5. Several conclusions can be derived from this scheme. It requires that chloramphenicol and F-Ery can both be bound simultaneously to the same ribosome, indicating that these antibiotics bind to two distinct sites. The presence of bound chloramphenicol leads to a change in the F-Ery binding site which results in a fourfold increase in the association constant of F-Ery to ribosomes. With three measured association constants the fourth equilibrium constant corresponding to the association of chloramphenicol to ribosomes containing F-Ery can be determined by the relationship:

$$\frac{K_a(\mathrm{R \cdot F\text{-}Ery + CM})}{K_a(\mathrm{R + CM})} = \frac{K_a(\mathrm{CM \cdot R + F\text{-}Ery})}{K_a(\mathrm{R + F\text{-}Ery})} \frac{[\mathrm{CM \cdot R \cdot F\text{-}Ery}][\mathrm{R}]}{[\mathrm{CM \cdot R}][\mathrm{R \cdot F\text{-}Ery}]} \quad (4)$$

Thus, the scheme of Figure 5 requires that the association constant of chloramphenicol with ribosomes be increased fourfold when F-Ery is present.

These conclusions derived from the scheme depicted in Figure 5 conflict with some previous work. Several studies using *E. coli* ribosomes (Pestka, 1974a; Pestka and Le Mahieu, 1974a; Wolfe and Hahn, 1965; Fernandez-Muñoz et al., 1971) and *Bacillus megaterium* ribosomes (Vazquez, 1966; Vazquez, 1963) concluded that chloramphenicol and Ery compete for the same site or overlapping sites on the ribosome. Other studies with *E. coli* ribosomes (Tanaka et al., 1966) and *B. subtilis* ribosomes (Taubman et al., 1966; Oleinick et al., 1968; Oleinick and Corcoran, 1969) suggest little or no interaction between these two antibiotics on the ribosome. Clearly these reports conflict with each other as well as with the results of

this study. There may be a number of explanations to account for these discrepancies.

One possibility is that a derivative of an antibiotic may have different binding properties from the parent compound. Table V illustrates that the absolute rate constants for [^{14}C]erythromycin and F-Ery binding to 70S particles are fairly different. However, our experiments indicate that F-Ery binds to the same site as erythromycin. This is also supported by the work of Vince et al. (1976) who found essentially the same equilibrium constant as we determined, and also found that, like erythromycin, F-Ery has one strong binding site per ribosome. It is still possible that the fluorescein moiety of F-Ery perturbs the ribosome so as to increase chloramphenicol binding affinity, whereas, in the absence of the fluorescein, Ery binding has the opposite effect. This explanation, however, does not explain the discrepancies between the other reports cited above.

A second possible explanation leads to a description of this system consistent with most of these reports. Oleinick and Corcoran (1969) have reported that erythromycin concentration higher than 10^{-5} M produces secondary binding to *B. subtilis* ribosomes with up to 7 molecules of Ery per ribosome at 10^{-3} M. This secondary binding appeared to be nonfunctional because it was also observed with ribosomes from erythromycin-resistant mutants. On Ery-sensitive ribosomes no increase was observed in the inhibition of polylysine synthesis when more than one Ery molecule per ribosome was bound. It has also been reported that polysomes from *E. coli* interact only weakly with Ery (Pestka, 1974a; Tai et al., 1974) but that high concentrations of Ery lead to nonspecific interactions with *E. coli* polysomes (Tai et al., 1974). Possibly, differences in the ribosomal preparations may account for some of the apparent discrepancies, for the presence of peptidyl-tRNA on ribosomes inhibits erythromycin binding (Pestka, 1974a).

In all of the reports cited above indicating competition between chloramphenicol and Ery, competition was observed only when the Ery concentration was greater than 10^{-5} M. This strongly suggests that the competition observed was due to secondary binding of Ery at or near the chloramphenicol binding site. Pestka (1974a) reported competition between these antibiotics on *E. coli* ribosomes, but no competition was observed when the Ery concentration was reduced to 10^{-6} M. We have calculated that at this concentration over 90% of the ribosomes should have bound Ery, indicating that chloramphenicol does not compete with Ery for its primary binding site. Further support for this conclusion is the report that on *E. coli* ribosomes high concentrations of Ery will totally displace chloramphenicol, but high concentrations of chloramphenicol will only partially displace Ery (Fernandez-Muñoz et al., 1971). Thus, we would tentatively conclude that Ery in its primary binding site is not displaced by chloramphenicol, but high Ery concentrations will cause secondary binding which is competitive with the chloramphenicol site. Consistent with these conclusions, Pestka and Le Mahieu (1974b) reported that some erythromycin derivatives inhibited chloramphenicol binding better than erythromycin binding. This observation was not consistent with a single site for binding of all the erythromycin derivatives to ribosomes, and, thus, they have concluded that some of the erythromycin derivatives were binding at additional sites rather than exclusively to the major erythromycin binding site. Our experiments were all done at F-Ery concentrations around 10^{-7} M, so no secondary binding would be expected.

One direct prediction of the scheme in Figure 5 is that chloramphenicol and Ery at low concentrations should enhance each others binding. Some preliminary direct binding experiments have been performed recently to examine this. Using flow dialysis, binding of chloramphenicol, at 1.22×10^{-5} to 1.63×10^{-5} M *E. coli* 70S ribosomes, was measured in the presence of from 0.16 to 10×10^{-5} M F-Ery. No evidence for enhanced chloramphenicol binding was seen. Instead an inhibition was observed. Assuming competitive binding, the apparent dissociation constant for F-Ery was computed to be about 7×10^{-6}. This is six times larger than the F-Ery dissociation constant measured by three different techniques. It suggests that the F-Ery molecule which inhibits chloramphenicol binding is not the same as the one which binds to the strongest erythromycin site. In parallel experiments no enhancement of total F-Ery binding by chloramphenicol could be observed. Unfortunately the lowest F-Ery concentrations used in the dialysis experiments correspond to the highest concentrations convenient to study by fluorescence. Thus, it is really not clear whether the discrepancies between fluorimetric studies of chloramphenicol-F-Ery interaction and other studies are yet resolved.

At present it seems safest to conclude that Ery and probably chloramphenicol as well have multiple binding sites on the 70S ribosome. The great experimental sensitivity of fluorescence allows one to work at sufficiently low concentration that only the strongest Ery site is populated to any significant extent. Several conclusions about this site seem established. Chloramphenicol does not bind to the strongest Ery binding site. This is consistent with a report that ribosomes from an Ery-resistant mutant of *B. subtilis* bind Ery only weakly, but chloramphenicol binding is the same as observed in the Ery sensitive strain (Oleinick et al., 1968). The presence of bound chloramphenicol enhances the binding of F-Ery to the strongest site. The binding enhancement we observe is quite small, however, so that it might be difficult to observe in experiments where Ery must be followed by radioisotopic techniques at concentrations where more than one Ery site is occupied. Our experiments also suggest that chloramphenicol affects Ery binding by inducing a conformational change on the ribosome.

Clearly the use of fluorescence to monitor kinetics and equilibrium is a powerful technique for elucidating the interaction between antibiotics and ribosomes. The inherent disadvantage of needing a fluorescent-labeled antibiotic is more than compensated for by the ability to work at low concentrations and the ability to monitor isolated steps involved in these interactions.

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Influence of DNA Structure on the Lactose Operator–Repressor Interaction†

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ABSTRACT: Kinetic studies of the complex formed between the lactose repressor and mung bean nuclease cleaved or sonicated λp*lac* DNA were consistent with our previous conclusion that the lactose repressor binding capacity is preferentially reduced by single-strand specific nucleases. Dissociation kinetics on nuclease-treated DNA revealed that mung bean nuclease damaged 30-40% of the operators. Fragmentation of the DNA either by nuclease or sonication decreased the rate of association between the repressor and the operator. Mapping experiments were performed by treating λp*lac* DNA with varying amounts of mung bean nuclease, cleaving with *Hin*II and *Hin*III and fractionating the fragments on 4% polyacrylamide gels. Statistical analysis of the loss of duplex restriction fragments as a function of nuclease concentration demonstrated that the *lac* operator fragment (720 base pairs) was not uniquely sensitive. However, when the operator-containing fragment was eluted from the gel, heat-denatured in 100% formamide, and analyzed on a 5% gel containing 98% formamide, a specific nick was revealed at approximately 100 nucleotides from the end. The amount of nicked fragment was commensurate with the extent loss of repressor binding. Control studies showed that the operator fragment was selectively nicked, since three other fragments of similar size were not nicked by the nuclease. This work is consistent with the notion that DNA contains conformationally unusual regions which may be involved in gene expression.

A goal of this laboratory is to determine if different regions of DNA chromosomes have different conformations and if these structures play a role in gene expression. Previous work on DNA polymers with defined repeating nucleotide sequences showed that the properties and conformation of a DNA were dictated by the sequence of nucleotides embodied by the polynucleotide chain (Wells and Wartell, 1974). DNAs possessing the same composition but different sequences had markedly different properties as studied by a variety of physical, chemical, spectroscopic, and biological techniques.

We have recently extended these investigations to a natural DNA (Chan and Wells, 1974). This work revealed that the lactose operator was especially sensitive to treatment with single-strand specific nucleases. Treatment of λp*lac* DNA with either S_1 or mung bean nuclease to give an average of 2-5 cuts maximally reduced its *lac* repressor binding capacity. However, an average of 300 cuts by any one of three nonspecific cutting agents (pancreatic DNase, micrococcal nuclease, or sonication) was necessary to give the same extent of reduction in repressor binding.

These studies were extended in order to better understand the cause and nature of the reduction of repressor binding after treatment with the single-strand specific nucleases. We have determined the rate of dissociation and association of the RO[1]

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[1] Abbreviations used are: RO, repressor-operator complex; *Hae, Haemophilus aegyptius; Alu, Arthrobacter luteus; Hin, Haemophilus influenzae;* IPTG, isopropyl thiogalactoside; ONPF, *o*-nitrophenyl fucoside; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; DTT, dithiothreitol; k_a, rate constant of association; k_d, rate control of dissociation.