0026-895X/89/030615-05$02.00/0

MOLECULAR PHARMACOLOGY, 36:615-619

Type of Inhibition of Peptide Bond Formation by Chloramphenicol Depends on the Temperature and the Concentration of Ammonium Ions

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Received April 17, 1989; Accepted June 23, 1989

SUMMARY

Using the same system that we used in a previous study [*Eur. J. Biochem.* **164**:53–58 (1987)], we have further examined the kinetics of inhibition of peptide bond formation by chloramphenicol in the puromycin reaction and we have applied conditions that are known to cause conformational changes to the 70 S ribosome. These conditions are the change in reaction temperature from 25° to 5° and the change in the concentration of NH_4^+ ion (50 mM versus 100 mM). The initial transient phase of competitive inhibition is now (100 mM NH_4^+ and 5° or 50 mM NH_4^+ and 25°) much more pronounced than at 100 mM NH_4^+ and 25°. Simple competitive inhibition is the only type of inhibition we can find when analyzing the kinetic information given by the initial slopes of the first-order time plots. This contrasts with the kinetics observed at 100 mM NH_4^+ and 25°, where a transient phase of competitive inhibition is followed (at higher concentrations of chloramphenicol) by a phase of mixed noncompetitive inhibition, which corresponds to a lower k_{cat} for peptidyltransferase (EC 2.3.2.12). This pattern of inhibition (competitive-mixed noncompetitive) was again obtained in this study using a ribosomal complex [acetyl[^{3}H]Phe-tRNA-poly(U)-ribosome] of low peptidyltransferase activity (k_{cat} = 0.91 min^{-1}), as was obtained previously when we used a complex of high activity (k_{cat} = 2.00 min^{-1}). Thus, the lowering of the k_{cat} of peptidyltransferase induced by chloramphenicol (from 0.91 to 0.34 min^{-1}) can occur irrespective of the activity status of peptidyltransferase. The conformational changes that are induced by chloramphenicol and lead to the lowering of the k_{cat} of peptidyltransferase need both relatively high (100 mM) concentrations of monovalent ion and higher temperature (25° as opposed to 5°). If these conditions are not met, the inhibition is simple competitive and the k_{cat} of peptidyltransferase remains unchanged. These results offer an explanation as to why a clear-cut competitive inhibition of the puromycin reaction by chloramphenicol has been difficult to observe for so many years.

Several studies have dealt with the kinetics of inhibition of peptide bond formation by CAM. The puromycin reaction has served as the prototype reaction for peptide bond formation and the ultimate objective has been the elucidation of the mechanism of action of CAM in protein synthesis. The kinetics of inhibition have been reported as competitive (1, 2), of the "mixed" type (3, 4), competitive and noncompetitive involving two binding sites (5), and competitive and mixed-noncompetitive, depending on the concentration of the inhibitor (6). Thus, the problem of whether CAM is a simple competitive inhibitor of the puromycin reaction remains unresolved. In analogy with the kinetics of inhibition of many enzymic reactions, a competitive inhibitor constitutes an important tool in the effort to decipher the mechanism of the enzymic reaction and the mechanism of action of the inhibitor. A more detailed analysis of the CAM problem is given in a previous publication (6).

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.

As we show in this paper, part of the discrepancy between the various reports on the kinetics of the inhibition by CAM may be due to the fact that the kinetics of the inhibition vary with the reaction temperature and the concentration of the monovalent ion used.

Materials and Methods

Materials and methods are those used in a previous publication (6), with the following additional details. Benzoylated DEAE-cellulose was purchased from Serva Feinbiochemica (Heidelberg, FRG). Heterogeneous tRNA from *Escherichia coli* strain W was purchased from Sigma and used for the preparation of Ac[^{3}H]Phe-tRNA (7) charged with 14.9 pmol of [^{3}H]Phe (82,000 cpm total)/A_{260} unit. This Ac[^{3}H]Phe-tRNA was treated essentially according to the method of Rheinberger *et al.* (8) in order to prepare the "treated" Ac[^{3}H]Phe-tRNA. Six hundred A_{260} units of the above (crude) Ac[^{3}H]Phe-tRNA and a column (1.2 × 25 cm) of benzoylated DEAE-cellulose were used. The treated Ac[^{3}H]Phe-tRNA had an increased specific radioactivity (850,000 cpm/A_{260}) because deacylated tRNAs were partly removed. This is evidenced by

ABBREVIATIONS: CAM, chloramphenicol; Ac[^{3}H]Phe-tRNA, *N*-acetyl-[^{3}H]-phenylalanyl-tRNA; complex C, the Ac[^{3}H]Phe-tRNA-poly(U)-ribosome ternary complex that bears Ac[^{3}H]Phe-tRNA bound to the ribosomal P-site; k_{cat}, catalytic rate constant.

the fact that complex C prepared with the treated Ac[^{3}H]Phe-tRNA had 84% of the bound donor in a puromycin-reactive state. The corresponding complex prepared with the crude Ac[^{3}H]Phe-tRNA had only 56% of the bound donor in a puromycin-reactive state. For the purpose of the present work, further removal of uncharged tRNA from the 'treated' Ac[^{3}H]Phe-tRNA was not necessary.

Preparation of ribosomes, ribosomal wash, and disc-adsorbed complex C. Washed ribosomes and the ribosomal wash were isolated from frozen *E. coli* B cells, as described previously (9).

Complex C was formed under two different conditions, depending on whether the ribosomal wash was present or absent during its formation.

In the presence of ribosomal wash, the disc-adsorbed complex C was prepared with crude Ac[^{3}H]Phe-tRNA [3.2 A_{260} units charged with 47.7 pmol of [^{3}H]Phe (262,000 cpm)/0.25 ml of incubation mixture], as described previously (9). The amount of Ac[^{3}H]Phe-tRNA bound to complex C, adsorbed in one half disc, was 8.9 pmol (49,000 cpm). Complex C contained 37.3% of the input Ac[^{3}H]Phe-tRNA and 8.5% of the ribosomes used. Over 99% of the disc-bound Ac[^{3}H]Phe-tRNA was reactive toward puromycin, i.e., it was bound to the ribosomal P-site.

In the absence of ribosomal wash, complex C was prepared as above but, instead of crude Ac[^{3}H]Phe-tRNA, treated Ac[^{3}H]Phe-tRNA was used [0.15 A_{260} units charged with 22.7 pmol of [^{3}H]Phe (125,000 cpm)/0.25 ml of incubation mixture]. The end point of the reaction of complex C with puromycin indicated that 84% of the bound donor (N_o) was converted to Ac[^{3}H]Phe-tRNA-puromycin (P). The values of $x = (P/N_o) \times 100$ were thus divided by 0.84, so that they can be fitted to the ordinate of the first-order time plot [ln $100/(100 - x)$ versus t] (see also Ref. 10).

Puromycin reaction. The puromycin reaction was carried out without preincubation (the disc-adsorbed complex C reacted with a mixture of puromycin and CAM at 5° or 25°) or after preincubation of the disc-adsorbed complex C with the inhibitor (20 min at 5° or 25°). In the presence of inhibitor, biphasic first-order time plots may be obtained, especially if enough reaction time is allowed. In these cases, the slope of the line going through the origin (initial slope of the time plot) is taken as the value of $k'_{obs} = k$ (e.g., Fig. 1). The maximal value (k_{max}) of k (e.g., Fig. 5) has been defined previously (9).

Results

The double-reciprocal plots of Figs. 1, 3, and 4 (see below) were based on first-order time plots (6), which, depending on the concentration of the inhibitor and of puromycin, may be biphasic, especially if enough reaction time is allowed. When this happens, only the kinetic information pertaining to the initial slopes is analyzed.

The kinetics of inhibition of the puromycin reaction were first examined at 5° and the results are shown in Fig. 1A. In contrast to the results obtained at 25° (6), increasing the concentration of the inhibitor did not alter the type of inhibition, which remained competitive up to high concentrations of inhibitor (20 K_i). At 5°, as expected, the k_{cat} of peptidyltransferase calculated from Fig. 1A is lower than at 25° (Table 1). The type of inhibition observed at 5° (Fig. 1A) is completely different from that observed at 25°. At 25° (Fig. 1B) and at low concentration of inhibitor, the inhibition is competitive, but after preincubation of complex C with the inhibitor the inhibition is not of the competitive type. At higher concentrations of inhibitor with or without preincubation, the inhibition is of the mixed-noncompetitive type. More data on the inhibition at 25° are given in Ref. 6. The results given in Fig. 1A are the same independently of whether complex C is preincubated with CAM. The K_i for the inhibition at 5° is 5.0 μM and is obtained

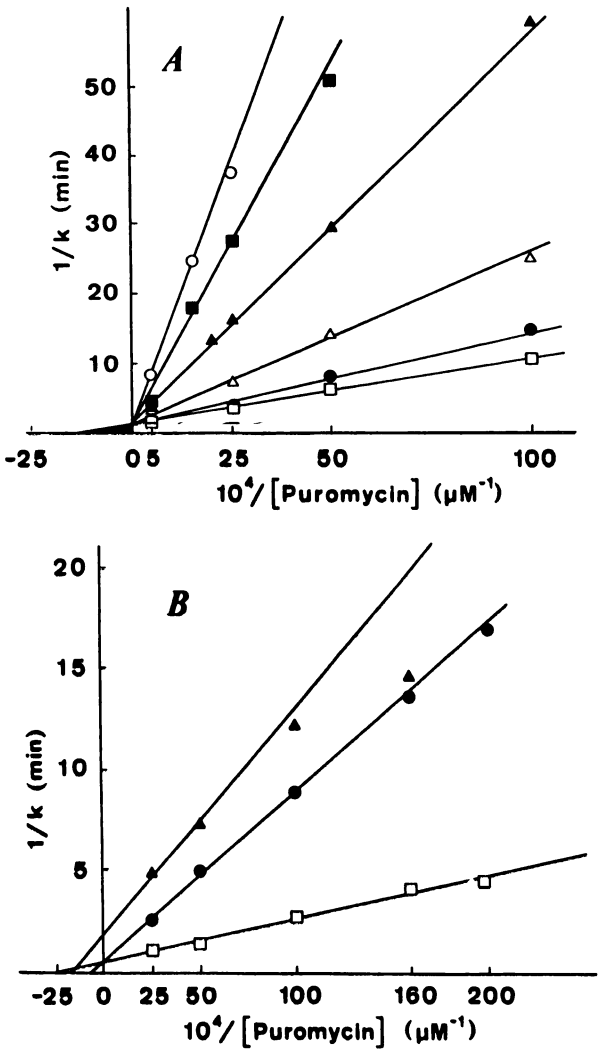

Fig. 1. Double-reciprocal plots of k values versus puromycin concentration in the absence or in the presence of CAM. A, □, The puromycin reaction was carried out at 5° and at 100 mM NH_4^+, in the absence of CAM. In the remaining experiments, the disc-adsorbed complex C was preincubated with various concentrations of CAM in reaction buffer containing 100 mM NH_4^+ at 5° and then puromycin was added to give the final concentrations indicated. CAM concentrations were 3 μM (●), 10 μM (△), 30 μM (▲), 70 μM (■), and 100 μM (○). B, □, The puromycin reaction was carried out at 25° and 100 mM NH_4^+ in the absence of CAM; ●, the disc-adsorbed complex C was added to a mixture of 3 μM CAM and puromycin at the indicated concentrations; ▲, the disc-adsorbed complex C was preincubated with 3 μM CAM in reaction buffer containing 100 mM NH_4^+, at 25° for 20 min, and then puromycin was added to give the final concentrations indicated.

from the slope replot shown in Fig. 2. The intercept with the slope axis gives the K_s/k_3 of the control and this shows that the inhibition is simple competitive.

Experiments where complex C was preincubated with CAM at 25° and then reacted with puromycin at 5° were also carried out. The type of inhibition was also simple competitive and plots like those of Figs. 1A and 2 were obtained. In the reverse experiments, complex C was preincubated with CAM at 5° and then the puromycin reaction was carried out at 25°. The inhibition exhibited two phases, as if the reaction with puromycin had been carried out at 25° from the start without preincubating complex C with CAM (6).

Because at 25° and at 100 mM NH_4^+ CAM appears to act as

TABLE 1

Equilibrium and kinetic constants derived from primary and secondary kinetic plots

The K_i and the k_3/K_s values given in the first and the third columns, were calculated from the slope replot. The K_i value in the middle column was calculated from the competitive double-reciprocal plot. The k_3 and K_s values were calculated from the double-reciprocal plot obtained in the absence of inhibitor. The k_3^* and aK_i^* values have been calculated from the intercept replot, on the assumption that, after preincubation of *C* with high concentrations of *I* and in the continuous presence of *I*, product comes only from *C**. On the same assumption, the K_i^* and the k_3^*/K_s^* values have been calculated from the slope replot. k_3 and k_3^* are taken as a measure of the k_{cat} of peptidyltransferase in *C* and *C**, respectively.

Parameter	Unit	Puromycin reaction at 5°, 100 mM NH_4^+, with ribosomal wash	Puromycin reaction at 25°, 100 mM NH_4^+, no ribosomal wash	Puromycin reaction at 25°, 50 mM NH_4^+, with ribosomal wash
k_3	min^{-1}	0.77	0.91	0.36
K_S	μM	485	444	666
k_3/K_s	min^{-1} mM^{-1}	1.6	2.0	0.54
k_3^*	min^{-1}		0.34	
K_s^*	μM		238	
k_3^*/K_s^*	min^{-1} mM^{-1}		1.4	
K_i	μM	5.0	0.86	1.2
K_i^*	μM		1.20	
aK_i^*	μM		17.00	

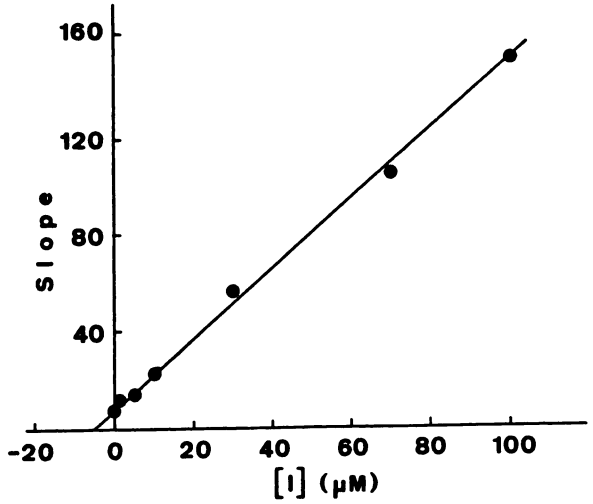

Fig. 2. Slope replot (slopes of double-reciprocal plots versus [I]). The data were obtained from the double-reciprocal plots of Fig. 1A after preincubation, at 5° and at 100 mM NH_4^+, with CAM.

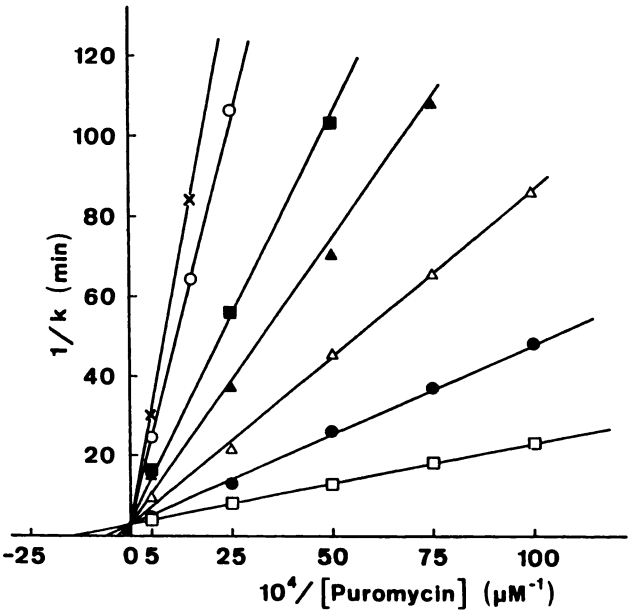

Fig. 3. Double-reciprocal plot ($1/k$ versus 1/[puromycin]) for the puromycin reaction (50 mM NH_4^+ and 25°) carried out with complex C that was prepared in the presence of ribosomal wash. □, In the absence of CAM; ●, preincubation with 1 μM CAM; △, preincubation with 3 μM CAM: ▲, preincubation with 6 μM CAM; ■, preincubation with 10 μM CAM; ○, preincubation with 20 μM CAM; ×, preincubation with 30 μM CAM.

a modifier (6), it was of interest to see whether such behavior would be repeated at a low NH_4^+ concentration. The results of experiments at 50 mM NH_4^+ are shown in Fig. 3. Under these conditions, CAM acts as a simple competitive inhibitor. The k_3/K_s and the K_i values, obtained from the slope replot, are given in Table 1.

The activity status of peptidyltransferase, as measured by the ratio k_3/K_s, appears to be low in both cases where simple competitive kinetics were observed (Figs. 1A, 2, and 3, Table 1). Thus, it might be argued that the low activity of peptidyltransferase per se is responsible for the competitive kinetics that we observe. For this reason, we reexamined the type of inhibition, at 25° and at 100 mM NH_4^+, using complex C of low activity instead of that with higher activity (k_{cat} = 2.0 min^{-1}) used previously (6). Such a complex was prepared in the absence of the ribosomal wash and with a preparation of Ac[^{3}H]Phe-tRNA from which uncharged tRNAs were partially removed. This complex had the donor bound mostly (84%) to the P-site. Its k_3/K_s value was indeed low (Table 1). However, the behavior of CAM was the same as that observed with complex C of a high k_3/K_s (6). Namely, a transient competitive phase (a figure analogous to Fig. 1B was obtained but it is not shown) and a mixed noncompetitive phase (Fig. 4) at higher concentrations of inhibitor were observed.

Corroborating evidence for the mixed noncompetitive phase comes from the linear Dixon plots (not shown). The intercept replot (Fig. 5), which is not linear, establishes the change in the k_{cat} of peptidyltransferase. The value for k_3^* (Fig. 6) was calculated from the extrapolated intercept value at zero concentration of inhibitor. The *I*-axis intercept of the same replot gives the aK_i^* value. More details on this type of inhibition are given in Ref. 6.

Discussion

In this as well as in a previous study (6), we have explored the kinetic information that is obtained from the early part of the first-order time plots (initial slopes). Analysis of this infor-

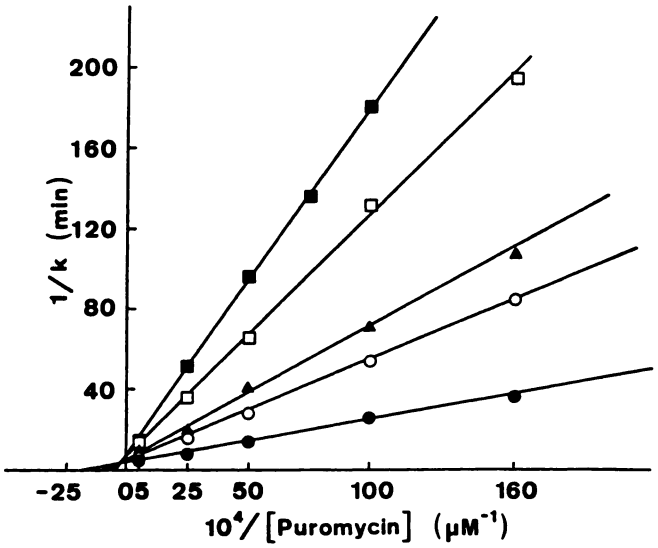

Fig. 4. Double-reciprocal plot (1/*k* versus 1/[puromycin]) for the puromycin reaction (100 mM NH_4^+ and 25°) carried out with complex C that was prepared in the absence of ribosomal wash and preincubated with various concentrations of CAM. CAM concentrations were 3 μM (●), 6 μM (○), 10 μM (▲), 20 μM (□) and 30 μM (■).

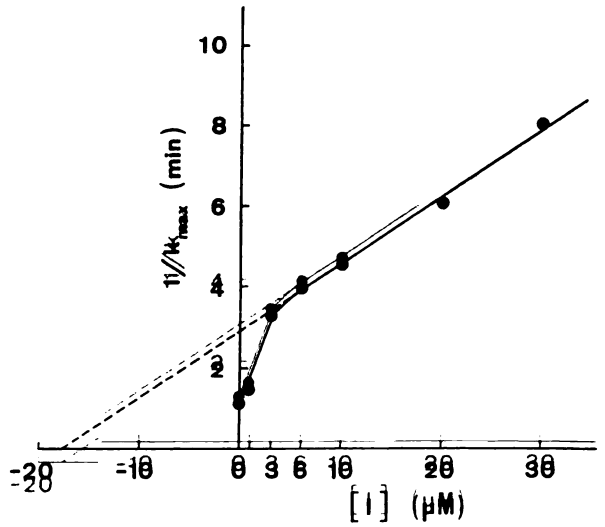

Fig. 5. Intercept replot ($1/k_{max}$ versus [*I*] ≡ CAM). The data were taken from the double-reciprocal plots of Fig. 4.

mation leads to two distinct patterns of inhibition by CAM. If we compare the pattern obtained at the higher temperature (25°) and at 100 mM NH_4^+, as opposed to 50 mM NH_4^+, we see that at 100 mM NH_4^+ a complex kinetic scheme (Fig. 6) is needed in order to interpret the CAM results. This kinetic scheme has been documented previously (6) as an "alternative model" and is presented here in Fig. 6 for the purposes of discussion. At 50 mM NH_4^+, only the left half of the kinetic scheme of Fig. 6 is needed, because we are dealing with simple competitive kinetics (Fig. 3). A similar distinction can be made if we compare the pattern of inhibition obtained at the higher ammonium concentration (100 mM) and at 25° versus 5°. At 25°, both the left and the right halves of the kinetic scheme of Fig. 6 are needed in order to explain the kinetics of inhibition (6). In contrast, at 5°, only the left half is needed, because here we are dealing again with simple competitive kinetics (Fig. 1A).

The results presented in this paper demonstrate that the

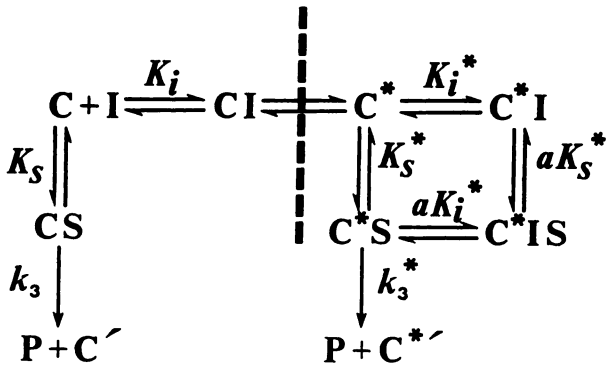

Fig. 6. Kinetic model for the puromycin reaction in the presence of CAM. *C*, complex C; *I*, CAM; *C**, modified complex C; *S*, puromycin; *P*, AcPhe-puromycin. *C'* or *C*'* represent the complexes after their reaction with puromycin. *Left half* of the kinetic scheme (competitive kinetics) is the part on the *left* of the *dotted line* (adapted from Ref. 6).

kinetics of inhibition of peptide bond formation by CAM vary when the conditions are changed. This may explain the fact that for many years it could not be settled whether puromycin (the substrate) is competitive with CAM (the inhibitor), as far as the kinetics of inhibition can tell.

The alternative conditions in temperature and ionic strength that we applied are known to affect the conformation of the ribosome. It is also known that the ribosome can exist in various conformations (11, 12) and that the peptidyltransferase domain is intimately associated with ribosome structure (13, 14). Thus, the results of this study can also constitute further evidence that, as proposed previously (6), CAM is involved in conformational changes in the peptidyltransferase domain. When, in the presence of CAM, these changes are allowed to occur, they cause a reduction in the k_{cat} of peptidyltransferase. If they are not allowed to occur, the k_{cat} of peptidyltransferase remains unchanged (competitive kinetics), as far as the initial slopes of the time plots are analyzed.

Using Fig. 6, we may speculate that, in this oversimplified kinetic scheme, some critical conformational changes must accompany the equilibrium $CI \rightleftharpoons C^*$ that is marked with the dotted line. Furthermore, data from the literature may be used so that we can speculate on the possible role of reaction temperature and NH_4^+ ions in allowing CAM to modify complex C to C^* (Fig. 6). The early report by Vogel *et al.* (15), which deals with the so-called "heat-dependent Zamir-Elson transition," directly implicates the NH_4^+ ion and the reaction temperature in conformational changes that occur in the peptidyltransferase domain. These changes were detected as changes in the activity of peptidyltransferase (fragment reaction) along with changes in the binding of CAM (15). A more recent report (16) documents the involvement of 16 S rRNA in the heat-dependent Zamir-Elson transition of the 30 S ribosomal subunit, as a function of monovalent or divalent cations. Further evidence that the monovalent cation K^+ is intimately associated with the function of Mg^{2+} ion in keeping the native conformation of 16 S rRNA in the 30 S ribosomal subunit has been also presented (17). We can speculate here that in the 50 S ribosomal subunit a heat-dependent Zamir-Elson transition involving 23 S rRNA may be needed before CAM can modify complex C. It should be noted that peptidyltransferase and the target of CAM have been localized within a region in domain V of 23 S rRNA (13, 18).

The function of NH_4^+ ion in the conformational changes

associated with the conversion of complex C to C^* may be similar to the function proposed for the K^+ ion in relation to conformational changes that occur in 16 S rRNA (17). In analogy with this proposal, we propose that the NH_4^+ ion (at 100 mM but not at 50 mM) destabilizes some regions of 23 S rRNA and makes it more "open" and accessible to the modifying effect of CAM. This destabilization of 23 S rRNA may involve the replacement of Mg^{2+} by NH_4^+ ions, which results in a decrease in the presumed cross-linking of Mg^{2+} ion on the rRNA molecule.

The results of the experiments carried out with complex C prepared in the absence of the ribosomal wash (Figs. 4 and 5) establish that, if the appropriate conditions prevail (25°, 100 mM NH_4^+), the modification of complex C by CAM can be observed regardless of whether the k_{cat} is high or low.

In conclusion, we have presented evidence that the kinetics of the inhibition of peptide bound formation by CAM depend on the conformational status of the ribosome and, thus, vary depending on the reaction conditions used. The kinetics can be those of simple competitive inhibition or those of a transient competitive inhibition, which at 25° is converted to mixed noncompetitive with increasing concentrations of CAM. Furthermore, we show that the conformational changes (lowering of the k_{cat}) induced by CAM can occur irrespective of the activity status of peptidyltransferase.

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