MODES OF ACTION OF ERYTHROMYCIN AND THIOSTREPTON AS INHIBITORS OF PROTEIN SYNTHESIS

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Received 10 August 1971
Revised version received 26 August 1971

1. Introduction

During protein synthesis in cell-free extracts of *Escherichia coli* peptidyl-tRNA is located on one of two ribosomal binding sites [1]. The nascent peptide is transfered from tRNA bound at the P site on to amino acyl-tRNA bound at an adjacent A site [2]. This reaction requires an enzyme, peptidyl transferase, which is an integral part of the 50 S subunit [3]. Before the peptide chain can elongate further, peptidyl-tRNA bound at site A must be translocated to site P to allow a further amino acyl-tRNA to attach at site A. Translocation requires GTP hydrolysis and 'G' factor [4].

Puromycin reacts with peptidyl-tRNA bound to site P to release peptidyl-puromycin [5, 6]. This reaction requires only the correct ionic environment and is catalysed by peptidyl transferase [7]. The puromycin reaction can thus be used to study the formation of an individual peptide bond. Translocation can also be detected using puromycin since more peptide moves to site P. Finally, the reaction assays the distribution of peptidyl-tRNA between sites A and P in a given ribosome population.

We have utilised the puromycin reaction to elucidate the mechanisms of action of two antibiotics – thiostrepton and erythromycin. Both have been proposed to inhibit translocation [8, 9]. Studies on washed ribosomes have suggested that thiostrepton, but not erythromycin, may inhibit translocation. After inhibition, by thiostrepton or erythromycin, of crude extracts actively synthesising protein, thiostrepton causes peptidyl-tRNA to accumulate in site P. Erythromycin apparently causes peptidyl-tRNA to accumulate in site A. We suggest that thiostrepton may act primarily by blocking the binding of amino acyl-tRNA to ribosomes. Erythromycin may inhibit translocation, but the possibility is not excluded that it could inhibit the enzyme peptidyl transferase.

2. Materials and methods

E. coli strain B163 was grown, harvested and frozen until use [10]. Crude extracts were prepared [11] and diluted to 10 mg/ml ribosomes.

Ribosome-free supernatant fraction was obtained from crude extracts [11], and was dialysed for 18 hr at 0–4° against 5 mM Tris-Cl buffer, pH 7.4, containing 10 mM magnesium acetate, 86 mM KCl and 6 mM mercaptoethanol, and stored frozen.

The reaction mixture for amino acid incorporation into protein has been described [11]. Incubations were at 30° for 5 min. Incorporations were directed by the natural messenger RNA of the extract.

The puromycin reaction was with ribosomes prepared by centrifugation of incorporation mixtures at 165,000 *g* for 45 min after dilution with 5 mM Tris-Cl buffer, pH 7.4, containing 10 mM magnesium acetate. Ribosome pellets were resuspended, to a concentration of 2 mg/ml, in 5 mM Tris-Cl buffer, pH 7.4, containing 10 mM magnesium acetate and 86 mM KCl. Ribosome suspensions (1 ml) were incubated for 5 min at 30° with or without puromycin (0.1 mM). For translocation studies, ribosomes were sedimented from incorporation mixtures, were washed by two cycles of centrifugation in 5 mM Tris-Cl buffer, pH 7.4, containing 10 mM magnesium acetate and 500

mM NH_4Cl, and were then resuspended in 5 mM Tris-Cl buffer, pH 7.4, containing 10 mM magnesium acetate and 86 mM KCl. Samples (1 ml) were incubated with puromycin in an additional reaction mixture (1 ml) as described under table 2.

The release of nascent protein from puromycin-treated ribosomes was estimated [11]. Results are expressed as percentage of nascent protein released from ribosomes, relative to 100% bound on control ribosomes.

^{14}C-labelled *Chlorella* protein hydrolysate (52 mCi/matom carbon) was obtained from the Radiochemical Centre (Amersham, England). Puromycin dihydrochloride was obtained from the Nutritional Biochemicals Corporation (Cleveland, Ohio). Chlortetracycline, erythromycin and thiostrepton were generously provided by Dr. Eric Cundliffe.

Table 1
Antibiotic effects on the puromycin reaction with washed ribosomes.

System	Protein released from ribosomes by puromycin (%)	Inhibition of the puromycin reaction (%)
Control ribosomes	35	–
Ribosomes + chlortetracycline	34.5	–
Ribosomes + thiostrepton	35.5	–
Ribosomes + erythromycin	31.5	10

Labelled ribosome suspensions (specific activity 15,000 dpm/mg) were prepared as described in sect. 2. Samples (1 ml) were incubated for 5 min at 30° with puromycin (0.1 mM). Release of nascent protein was determined as described in sect. 2. The effects, on the reaction, of chlortetracycline, thiostrepton and erythromycin at concentrations of 250 μg/ml, were determined.

3. Results and discussion

Thiostrepton and erythromycin are potent inhibitors of protein synthesis in *E. coli* both *in vivo* and *in vitro*. We have used chlortetracycline as a control inhibitor – it inhibits the codon-directed binding of amino acyl-tRNA to ribosomes [12]. We can thus relate results obtained with an antibiotic of known mechanism of action to results obtained with an antibiotic of unknown action.

We have determined if the three antibiotics used here inhibit the puromycin reaction with ribosome-bound nascent peptides formed under the influence of natural messenger RNA. The results are presented in table 1. Labelled ribosome suspensions were incubated with puromycin in the presence or absence of chlortetracycline, thiostrepton or erythromycin and the amounts of nascent protein released were determined. The three inhibitors were present at very high concentration (250 μg/ml) – a five-fold excess over the level of puromycin used (50 μg/ml). These high concentrations were chosen deliberately to bias the experiment in favour of inhibition. Neither chlortetracycline nor thiostrepton inhibited the puromycin reaction. With erythromycin, the puromycin reaction was inhibited to a very small, but consistent, extent (10%). Washed ribosomes, as prepared for this experiment, have peptidyl-tRNA bound to either site A or site P of the ribosome. As seen from table 1, puromycin releases 35% of the bound nascent peptides. Since the reaction needs only the correct ionic environment, only those ribosomes with peptidyl-tRNA bound at site P react with puromycin. Any inhibition of the reaction under these conditions represents an inhibition of peptidyl transferase – an inhibition characteristic of several antibiotics [11, 13]. Clearly, however, peptidyl transferase is not inhibited directly by either chlortetracycline or thiostrepton under our conditions. Since chlortetracycline acts on the 30 S ribosomal subunit, it should not inhibit the puromycin reaction which is a property of the 50 S subunit. Our results support this interpretation and agree with those of others [9].

Erythromycin is particularly interesting. It inhibits peptidyl transferase to only a very small extent, and this inhibition could be artefactual. Under certain conditions, *in vivo*, erythromycin does not significantly inhibit the puromycin reaction [9] and other workers [13] have claimed that, *in vitro*, peptidyl transferase is not inhibited by erythromycin. However, Tanaka et al. [14] have postulated that erythromycin selectively inhibits the puromycin reaction with ribosome-bound phenylalanyl-phenylalanyl-tRNA. This situation will be discussed later in this paper.

We have tested the three antibiotics for their ability to inhibit translocation. Labelled ribosomes, sedimented

Table 2
Antibiotic effects on the puromycin reaction with NH_4Cl-washed ribosomes in the presence of GTP and supernatant fraction.

System	Protein released from ribosomes by puromycin (%)
(1) Control ribosomes	36
(2) Ribosomes + reaction mixture minus supernatant fraction	35
(3) Ribosomes + reaction mixture	65
As for (3) + chlortetracycline	66
As for (3) + thiostrepton	40
As for (3) + erythromycin	60

Ribosomes were washed with NH_4Cl as described in sect. 2. 1 ml samples (specific activity 13,000 dpm/mg) were incubated for 5 min at 30° with puromycin (0.1 mM) in the presence of a reaction mixture (1 ml) containing 5 μmole phosphoenolpyruvate, 1 μmole ATP, 0.15 μmole GTP, 50 μg pyruvate kinase, 6 μmole mercaptoethanol and 0.2 ml supernatant fraction in 5 mM Tris-Cl buffer, pH 7.4, containing 10 mM magnesium acetate and 86 mM KCl. As under table 1 chlortetracycline, thiostrepton or erythromycin were added, and the effects on the release of protein studied.

Table 3
Inhibition of ribosomes in the puromycin reaction by pretreatment of crude extracts with antibiotics during protein synthesis.

System	Protein released from ribosomes by puromycin (%)
Control ribosomes	36
Ribosomes from extracts treated with chlortetracycline	55
Ribosomes from extracts treated with thiostrepton	57
Ribosomes from extracts treated with erythromycin	14

Incorporation mixtures were incubated for 5 min at 30°. One control sample (1.5 ml) was chilled on ice and to three other samples (1.5 ml) was added 500 μg of chlortetracycline, thiostrepton or erythromycin. Incubation was continued for 5 min before chilling. The samples were diluted with ice-cold 5 mM Tris-Cl buffer, pH 7.4, containing 10 mM magnesium acetate and the ribosomes were sedimented by centrifugation at 165,000 *g* for 45 min. The pellets were resuspended, in 5 mM Tris-Cl buffer, pH 7.4, containing 10 mM magnesium acetate and 86 mM KCl, to a concentration of 2 mg/ml and assayed in the puromycin reaction as for table 1. Specific activity of ribosomes was 15,000 dpm/mg.

from an incorporation mixture, were washed with a buffer containing NH_4Cl as described in sect. 2. This treatment removes any bound G factor. Puromycin reacts with ribosomes, washed in this way, to release peptides from peptidyl-tRNA bound at site P. For translocation to occur, these ribosomes need added G factor and GTP. Theformer is present in ribosome-free supernatant fractions. Ribosomes prepared as above were incubated with GTP, supernatant fraction and puromycin, in the absence or presence of the three antibiotics. Nascent protein release was estimated and the results are shown in table 2. Addition of GTP and supernatant fraction to control ribosomes almost doubles the stripping action of puromycin by effecting translocation of peptidyl-tRNA from site A to site P. The maximal stripping obtained (approximately 70%) is characteristic of the puromycin reaction under these conditions. Although in theory 100% stripping should be observed this level is never reached for reasons which have never been satisfactorily explained.

Table 2 shows that chlortetracycline has no effect on the puromycin reaction in the presence of GTP, and as discussed earlier this result is expected. Erythomycin exerts a small inhibitory effect on the puromycin reaction under these conditions. This is consistent with the results illustrated in table 1 and presumably represents a small inhibition of peptidyl transferase. Thiostrepton inhibits the puromycin reaction in the presence of GTP and G factor very markedly. Since thiostrepton does not inhibit the puromycin reaction directly (table 1) it seems that the antibiotic inhibits translocation under the above conditions. Although this interpretation agrees with the conclusions of others [8], it is at variance with recent work by Cundliffe [15].

We have used the three antibiotics to inhibit *E. coli* cell-free extracts which are actively synthesising protein, to attempt to block peptidyl-tRNA in

either site A or site P. Incorporation mixtures were prepared and incubated. After 5 min a control incorporation was chilled and to three others chlortetracycline, thiostrepton or erythromycin were added. Incubations were continued for 5 min before chilling. The samples were diluted and the ribosomes sedimented, resuspended and assayed in the puromycin reaction with no added GTP. The results are shown in table 3. Control ribosomes release the expected amount of nascent protein. Ribosomes from extracts inhibited with either chlortetracycline or thiostrepton behave similarly in the puromycin reaction, and amounts of nascent protein released were markedly higher than for control ribosomes, approaching the levels normally released by puromycin in the presence of GTP and supernatant fraction (cf. table 2). With ribosomes from extracts inhibited with erythromycin the reverse was true. The amount of nascent protein released was markedly less than from control ribosomes.

This result with erythromycin in unexpected. It failed to inhibit translocation under our conditions and was a poor inhibitor of the puromycin reaction itself. However, if the antibiotic is added to a system synthesising protein the ribosomes accumulate peptidyl-tRNA in a state where it is unable to react with puromycin (table 3). This effect would be expected of a translocation inhibitor which would allow peptide transfer but would restrict peptidyl-tRNA to site A, where it would be unable to react with puromycin. This result agrees with that of Cundliffe and McQuillen [9] who concluded that erythromycin inhibited translocation. Furthermore, the antibiotic inhibits the release of deacylated-tRNA, from ribosomes, which normally occurs during translocation [16]. Possibly, erythromycin can inhibit translocation only when added to ribosomes actively engaged in protein synthesis (table 3). Addition to a fractionated and more artificial system (table 2) may not favour inhibition. With washed ribosomes with G factor and excess GTP a translocation inhibitor would have to act extremely efficiently since a single translocation event would cancel any inhibitory effect.

There is a further possibility. If chloramphenicol is used in the experiment of table 3 an identical result to the one using erythromycin is obtained [11]. Chloramphenicol does not inhibit translocation but binds to peptidyl transferase and greatly inhibits this enzyme in the puromycin reaction. This reaction with washed ribosomes (conditions of table 1) is also inhibited by chloramphenicol, but to a smaller extent (cf. erythromycin). Since erythromycin and chloramphenicol compete for binding sites on the 50 S subunit [17] possibly the two antibiotics act similarly. Erythromycin may inhibit peptidyl transferase by binding to a sensitive site on the enzyme only under conditions of protein synthesis. This could explain why erythromycin is a poor inhibitor of the puromycin reaction with washed ribosomes (table 1). Alternatively, erythromycin may only inhibit the enzyme efficiently when polypeptide chains of certain amino acid composition and chain length are bound on the ribosomes. Washed ribosomes carrying a heterogeneous population of protein chains may be poorly inhibited (table 1). Conversely, blockage of peptidyl transfer during protein synthesis (table 3) may select conditions for maximal inhibition by the antibiotic. This interpretation is based upon recent work by Tanaka et al. [14].

In table 3 the result obtained with chlortetracycline is expected. The antibiotic prevents binding of amino acyl-tRNA to site A but inhibits neither the puromycin reaction with washed ribosomes nor translocation studied under our conditions. Ribosomes inhibited during protein synthesis by chlortetracycline should accumulate peptidyl-tRNA in site P since normal peptidyl transfer is prevented.

The result with thiostrepton is surprising. Under certain conditions thiostrepton inhibits translocation. Ribosomes inhibited during protein synthesis by thiostrepton should, therefore, accumulate peptidyl-tRNA in site A. In our experiments this is clearly not the case although again this result agrees with recent work by Cundliffe [15]

The similar behaviour of thiostrepton and chlortetracycline (table 3) is significant. Modolell (personal communication) claims that thiostrepton blocks binding of alanyl-tRNA to site A of ribosomes, under certain conditions. Our results, and those of Cundliffe [15], could indicate that inhibition of amino acyl-tRNA binding to ribosomes is the primary site of action of thiostrepton during inhibition of protein synthesis both *in vivo* and *in vitro*. As with chlortetracycline this inhibition blocks peptidyl-tRNA at site P. As an inhibitor of protein synthesis thiostrepton may never need to inhibit translocation, although undoubtedly such an inhibition can occur under selected artificial conditions *in vitro*.

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