THE MECHANISM OF CYCLOHEXIMIDE INHIBITION OF PROTEIN SYNTHESIS IN RABBIT RETICULOCYTES

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Cycloheximide blocks the movement of peptidyl-tRNA from acceptor (aminoacyl) site to the donor (peptidyl) site on reticulocyte ribosomes. This translocation reaction is dependent upon the transfer enzyme, TF-II, and GTP hydrolysis. Cycloheximide has no effect on the ribosome dependent GTPase activity of TF-II or on the peptidyl transferase reaction by which peptides on tRNA in the donor ribosomal site are transferred to an amino acid on tRNA in the acceptor site.

The glutarimide antibiotic, cycloheximide, inhibits cell free protein synthesis in systems derived from certain yeasts, plants and vertebrates by inhibition of the transfer of amino acids from aminoacyl-tRNA to nascent proteins on ribosomes (1). Recent developments in the fractionation and assay of the rabbit reticulocyte transfer system has made it possible to carry out separately each of the reactions recognized as steps of protein synthesis in this system. This fractionated transfer system has been used in the study reported here to determine the specific reaction of peptide synthesis that is inhibited by cycloheximide.

Materials and Methods: Cycloheximide was a gift from Dr. G.B. Whitfield of The Upjohn Company. Sparsomycin was obtained from Dr. A.R. Stanley of the National Cancer Institute. Fusidic acid was a gift from Dr. N. Tanaka of Tokyo University. Ribosomes, aminoacyl-tRNA, and the transfer enzymes (binding enzyme, and TF-II) were prepared as previously described (2).

The ribosome dependent hydrolysis of GTP by TF-II was measured as previously described (2). Incorporation of leucine from leucyl-tRNA into

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nascent globin peptides and the reaction of puromycin with ribosome bound aminoacyl-tRNA was measured as described in legends to the Tables.

Results: Table 1 compares the effect of cycloheximide, sparsomycin, and fusidic acid on the incorporation of leucine from leucyl-tRNA into globin peptides initiated in intact reticulocytes. Under the conditions used, incorporation is highly dependent on both of the transfer enzymes and GTP. All reaction mixtures were carried through two cycles of incubation. The first reaction mixtures contained TF-II when indicated. Nascent peptides are moved to the donor ribosomal site by the reaction promoted by TF-II under the incubation conditions used. After this incubation, binding en-

Table 1. The effect of the antibiotics on the incorporation of leucine into nascent globin peptides.

Additions 1st incubation	Additions 2nd incubation	leucine incorporated pmoles	% inhibition
none	TF-II	7.2	--
TF-II	none	7.2	--
none	cycloheximide (10 mM) TF-II	2.4	67
TF-II	cycloheximide (10 mM)	4.4	39
none	sparsomycin (6 μM) TF-II	2.8	61
TF-II	sparsomycin (6 μM)	2.8	61
none	fusidic acid (0.8 mM) TF-II	3.6	50
TF-II	fusidic acid (0.8 mM)	6.0	17

The first incubation contained in a total of 0.25 ml: 0.06 M tris-HCl, pH 7.5; 4 mM $MgCl_2$; 70 mM KCl; 0.01 M dithiothreitol; 0.20 mM GTP; and 0.50 mg ribosomes bearing nascent globin peptides. These ribosomes were prepared and washed as previously described (2). The mixture was incubated at 37° for 15 minutes. Additional salts, 75 pmoles ^{14}C-leucyl-tRNA (1050 pmoles leucine/mg tRNA), 4 μg TF-I, 0.008 mmoles NaF, and the indicated antibiotic was added. TF-II (4 μg) was added to the first or second incubation where indicated. After an incubation of 20 minutes at 37°, incorporation of ^{14}C-leucine into hot trichloroacetic acid insoluble peptides was measured as previously described (2).

Table 2. The effect of cycloheximide and sparsomycin on the reaction of puromycin with nonenzymatically bound phenylalanyl-tRNA.

Additions 1st incubation	Additions 2nd incubation	Phenylalanyl-puromycin pmoles phe	% inhibition
none	TF-II	3.7	--
TF-II	none	3.6	--
none	cycloheximide (1 mM) TF-II	2.0	45
TF-II	cycloheximide (1 mM)	3.7	0
none	sparsomycin (6 μM) TF-II	1.2	68
TF-II	sparsomycin (6 μM)	1.2	68

The first incubation contained in a total volume of 0.25 ml: 0.06 M Tris-HCl, pH 7.5; 4 mM $MgCl_2$; 70 mM KCl; 0.01 M dithiothreitol; 0.20 mM GTP; 0.50 mg ribosomes carrying 8.0 pmoles phenylalanyl-tRNA. TF-II protein (4 μg) was added to the first or second incubation where indicated. The ribosomes carrying nonenzymatically bound phenylalanyl-tRNA were prepared as previously described (3). After an incubation of 15 minutes at 37°, additional salts, 0.30 μmoles puromycin, and the indicated antibiotic were added to make a total volume of 0.50 ml. After a second incubation of 20 minutes at 37°, the puromycin product was extracted from the reaction mixture with 2.0 ml ethyl acetate after the addition of 1.0 ml 2 N ammonium bicarbonate, pH 9.0. One ml of the organic phase was counted by liquid scintillation.

Table 3. The effect of the antibiotics on the ribosome dependent GTPase of TF-II.

Inhibitor	GTP hydrolyzed pmoles	% inhibition
None	50	0
Cycloheximide (1 mM)	52	0
Sparsomycin (6 μM)	52	0
Fusidic Acid (0.2 mM)	20	60

zyme and leucyl-tRNA were added to the chilled reaction mixture along with TF-II and antibiotics, as indicated, then the reaction mixture was incubated

for a second time. Cycloheximide inhibited incorporation by 67% when it was added with TF-II for the second incubation. However, inhibition was greatly reduced when the TF-II catalyzed reaction was carried out during the first incubation prior to introduction of cycloheximide. This is in contrast to the effect of sparsomycin which inhibits the transfer of the peptide to aminoacyl-tRNA or puromycin (4). Under these experimental conditions, fusidic acid and cycloheximide appeared to have a similar effect. Inhibition by fusidic acid also was largely overcome if the TF-II reaction was carried out during the first incubation before the antibiotic was added to the system. Inhibition of leucine incorporation by cycloheximide and fusidic acid in systems in which the TF-II reaction had been previously carried out in the first incubation may be attributed to a second round of TF-II catalyzed translocation after incorporation of an amino acid during the second incubation. In separate experiments cycloheximide, sparsomycin and fusidic acid at these concentrations have been shown to have no detectable effect on binding enzyme activity as measured by enzymatic binding or ribosome and aminoacyl-tRNA dependent hydrolysis of GTP.

Table 2 compares the effect of cycloheximide and sparsomycin on the reaction of puromycin with ribosome bound phenylalanyl-tRNA. The formation of any phenylalanylpuromycin product stringently requires TF-II and GTP in this system (3). For these experiments, ribosomes bearing nonenzymatically bound phenylalanyl-tRNA were incubated with TF-II in either the first or second of a series of two incubations. Puromycin and one of the other three antibiotics were added as indicated for the second incubation. If the TF-II catalyzed reaction is carried out in the first incubation prior to the introduction of cycloheximide, the inhibitory effect of the antibiotic is completely lost. In contrast, sparsomycin continues to inhibit the formation of phenylalanylpuromycin even though the TF-II catalyzed reaction was carried out during the first incubation. We interpret this to mean that cycloheximide blocks the TF-II dependent movement of the

aminoacyl-tRNA from a puromycin unreactive site, the acceptor site, to a puromycin reactive site, the donor site, on the ribosomes. Sparsomycin appears to inhibit the reaction by which peptides or aminoacids in the donor ribosomal site are transferred to an amino acid or puromycin, in that movement of the aminoacyl-tRNA from unreactive to a reactive site does not reduce the inhibitory effect of sparsomycin. These results with sparsomycin are in agreement with earlier work (4). Cycloheximide has been shown to have no effect on the reaction with puromycin of various N'-acylaminoacyloligonucleotide fragments from tRNA to give the N'-acylaminoacylpuromycin product using a 60S ribosomal subunit from yeast.[1] This reaction is thought to be a direct measurement of peptidyl transferase activity responsible for peptide bond formation per se in protein synthesis (5).

TF-II has been shown to be a ribosome dependent GTPase (6). Table 3 further characterizes the site of inhibition of cycloheximide by demonstrating its lack of effect on TF-II catalyzed hydrolysis of GTP. Cycloheximide and sparsomycin have no effect on this GTPase activity. In contrast, fusidic acid strongly inhibits TF-II GTPase activity and appears to block the TF-II dependent step of peptide synthesis by this inhibition (7).

Discussion: These studies indicate that cycloheximide inhibits the translocation of aminoacyl or peptidyl-tRNA from the acceptor or aminoacyl site (puromycin unreactive) to the donor or peptidyl site (puromycin reactive) on ribosomes. The antibiotic might inhibit movement of aminoacyl-tRNA or entry into the peptidyl site.

The data provide further evidence that translocation of aminoacyl-tRNA and GTP hydrolysis related to TF-II activity may be uncoupled into separate steps. Translocation between the acceptor and donor ribosomal sites may be blocked with cycloheximide with no apparent effect on GTP hydrolysis by TF-II. In contrast, fusidic acid appears to inhibit this

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[1] Staehelin, T., personal communication

translocation by direct inhibition of TF-II GTPase activity.

The cycloheximide sensitive site on 80S ribosomes from yeast has been shown to be on the 60S ribosomal subunit (8). Cycloheximide has been shown to inhibit both chain initiation and chain extension in intact reticulocytes (9). Although the studies presented here do not directly characterize the ribosomal site of cycloheximide inhibition, the data suggest that the sensitive site may be the donor site on the 60S ribosomal subunit and that this site may be directly involved in polypeptide chain initiation as well as chain extension in rabbit reticulocytes.

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