CYCLOHEXIMIDE RESISTANCE IN YEAST: A PROPERTY OF THE 60s RIBOSOMAL SUBUNIT*

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Cycloheximide, a glutarimide antibiotic isolated from extracts of Streptomyces griseus, inhibits protein biosynthesis in certain yeast, protozoa, plant and mammalian cells (for review, cf. Sisler and Siegel, 1967). Cycloheximide acts at the transfer step in protein synthesis, subsequent to the formation of the ternary complex between mRNA, aminoacyl-tRNA and the ribosome (Siegel and Sisler, 1964).

Cell-free extracts prepared from various species of Saccharomyces differ widely in their sensitivity to cycloheximide. Protein biosynthesis catalyzed by extracts of S. fragilis is unaffected by cycloheximide (Siegel and Sisler, 1965) while similar preparations of S. cerevesiae (Cooper et al., 1967), S. elipsoideus (Widuczynski and Stoppani, 1965) and S. pastioranus (Siegel and Sisler, 1964) are sensitive to the antibiotic. Gene-controlled resistance to cycloheximide (Middlekauff et al, 1957; Wilkie and Lee, 1965) has been demonstrated in cell-free preparations from strains of S. cerevesiae treated with ultraviolet light (Cooper et al., 1967).

In the present report, ribosomes and supernatant enzyme isolated

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from resistant and sensitive strains of Saccharomyces have been used to study cycloheximide resistance. The data indicate that resistance and sensitivity to cycloheximide are properties associated with the 60s ribosomal subunit.

MATERIALS AND METHODS

Cycloheximide was obtained from Nutritional Biochemicals, ^{14}C-L-phenylalanyl-tRNA from New England Nuclear and D_2O from Bio-Rad Laboratories. The source of other substrates and inhibitors used in this study has been recorded in previous publications (Grollman, 1966, 1967a, 1967b). S. cerevesiae (E1813, from the collection of Dr. Ben-Zion Dorfman) and S. fragilis, (ATCC-10022) were grown with aeration at 30°C in proteose-peptone beef extract medium (0.1% bacto-beef extract-1% Difco proteose peptone No. 3-0.5% NaCl-1% glucose), adjusted to pH 5.0 with HCl. Growth was followed by measuring optical density in a Klett-Summerson colorimeter at 400-465 mμ. Cells were harvested at an optical density of 100 Klett units and washed with a solution composed of 0.01 M imidazole, pH 7.0; 0.02 M NH_4Cl, 0.004 M magnesium acetate and 5×10^{-4} M β-mercaptoethanol (Buffer A).

Crude ribosomes were isolated by the method of Downey et al. (1965). 80s ribosomes were isolated from sucrose density gradients as described in the legend to Figure 2. One mg of ribosomes was assumed to give an OD_{260} of 9.3. Initial rates of polyphenylalanine synthesis from ^{14}C-phenylalanyl-tRNA were measured in cell-free extracts as previously described (Grollman, 1967a). Cycloheximide was added just prior to ^{14}C-phenylalanyl-tRNA, which was added last. Components of the standard reaction mixture, described in the legend to Figure 1, were incubated at 25° for 20 minutes and the reaction terminated by the addition of cold 10% trichloroacetic acid (TCA). ^{12}C-L-phenylalanine was added to a concentration of 2×10^{-4} M and the reaction mixtures heated at 95° for fifteen minutes. After chilling for 15 minutes in an ice bath,

precipitates were collected by filtration on Millipore filters, washed three times on the filter with 5% TCA, glued to planchets and dried. Radioactivity was determined in a Nuclear Chicago low-background gas flow counter with an efficiency of 21%. The concentration of cycloheximimide necessary to inhibit polyphenylalanine synthesis by 50% was determined graphically by plotting percent inhibition versus the logarithm of the molar concentration.

60s and 40s ribosomal subunits were prepared by suspending the crude ribosomes in Buffer A and dialyzing them (Chao, 1957) for 15 hours against a solution of 0.01 M potassium phosphate, pH 7.5 and 5×10^{-4} M mercaptoethanol. Sucrose density gradient centrifugation (Britten and Roberts, 1960) was carried out as described in the legend to Figure 2.

RESULTS

Effect of cycloheximide on the transfer reaction: Inhibition of phenylalanine transfer from phenylalanyl-tRNA into polyphenylalanine by various concentrations of cycloheximide is illustrated in Figure 1. Polyphenylalanine synthesis catalyzed by extracts of S. cerevesiae was inhibited by 50% at a concentration of 10^{-5} M and by 75% at a concentration of 10^{-4} M. In contrast, extracts of S. fragilis were resistant to the effects of cycloheximide at a concentration of 10^{-4} M and were inhibited only by 30 percent at a concentration of 10^{-3} M.

Extracts prepared at different times from the same frozen batch of S. cerevesiae cells showed rather similar sensitivity to the antibiotic but some variability was noted when batches of cells prepared at different times were compared. Reconstituted 80s ribosomes required somewhat higher concentrations of cycloheximide to obtain a 50% inhibition of peptide synthesis (Table II).

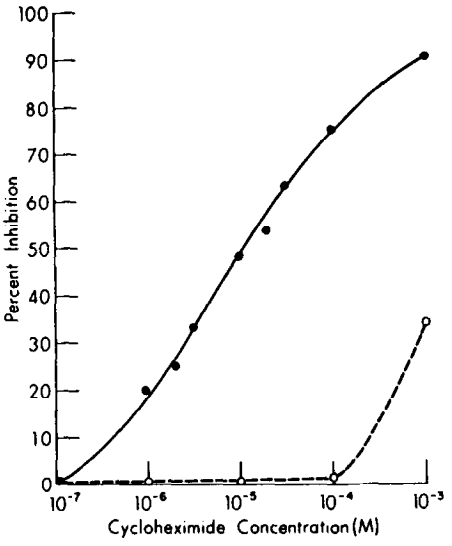

Figure 1. Inhibition of the transfer reaction by cycloheximide. Incubation mixtures contain, in a volume of 1.0 ml, 100 µmole of imidazole, pH 7.0; 6 µmoles of magnesium acetate; 100 µmoles of ammonium chloride; 0.8 µmoles of spermine; 2 µmoles of GTP; 80 µg of polyuridylic acid; 28 µg of ^{14}C-phenylalanyl-tRNA (2,200 cpm); 1 OD_{260} unit of ribosomes and the indicated final concentration of cycloheximide. In the experiments with S. cerevesiae ribosomes, 40 µl of a freshly prepared 100,000 x g supernatant enzyme fraction was also added. Reaction mixtures were incubated for 20 minutes at 25°C and prepared for the determination of radioactivity as described under "Methods". ●—● S. cerevesiae; O--O S. fragilis.

Dissociation of 80s ribosomes: A number of conditions were tested for the dissociation of the 80s ribosomes into the 60s and 40s subunits, including prolonged dialysis against buffers containing 10^{-4} M or 10^{-5} M magnesium acetate or dialysis in the presence of EDTA (Chao, 1957). In most cases, there was considerable loss of biosynthetic activity with a release of low molecular weight RNA which could not be prevented by the use of bentonite. The conditions finally selected (cf. Materials and Methods) allowed 70-90% of the 80s particles to dissociate with minimal degradation (Fig. 2) and with maximal recovery of activity on reassociation. If the magnesium concentration was adjusted to 10^{-2} M immediately follow-

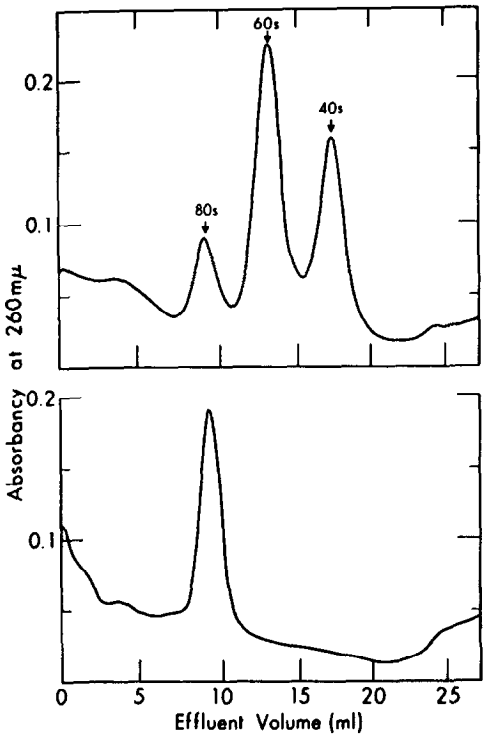

Figure 2. Sucrose density gradient centrifugation of dissociated ribosomes from S. fragilis. Upper: 4 OD_{260} units of dissociated ribosomes, suspended in 1 ml of 5 x 10^{-4} M imidazole, pH 7.0, 10^{-4} M magnesium acetate and 5 x 10^{-4} M mercaptoethanol, were layered on 26 ml of a 5-20% sucrose gradient prepared in the same buffer. Samples were centrifuged at 30,000 rpm for 120 minutes in a fixed angle rotor. Optical density at 260 mμ was determined during the collection of the gradient by means of a flow cell with a 5 mm light path attached to a Gilford Spectrophotometer. Lower: 4 OD_{260} units of undissociated ribosomes treated in an identical manner except that the concentration of magnesium acetate was increased to 10^{-2} M. Average sedimentation velocity constants and purity of 80s ribosomes and subunits was determined by recentrifugation in sucrose density gradients and by band centrifugation through 96% D_2O (Vinograd et al., 1963), employing a model E analytical ultracentrifuge equipped with UV optics. For the latter determinations, 30 μl of a solution of ribosomal subunits (1-2 OD_{260} per ml), were placed in the sample well of a band-forming centerpiece and centrifuged at 29,500 rpm at 16°C through 0.69 ml of a 96% D_2O solution containing 5 x 10^{-4} M imidazole, pH 7.0 and 10^{-4} M magnesium acetate. The $s_{20,w}$ values indicated in this Figure represent the sedimentation constants estimated from the relative position of the 70s, 50s and 30s ribosomal particles prepared from E. coli which were sedimented at the same time in similar sucrose density gradients. Confirmation of these values was obtained by the band centrifugation technique. A partial specific volume of 0.63 was assumed for both kinds of subunits. Values for the density and viscosity of water and deuterium oxide were taken from the International Critical Tables, v. VII and from Kirshenbaum (1951). Under the conditions employed, a 5% contamination of the purified ribosomes or subunits could have been detected.

Table I

Relative Activity of Yeast Ribosomes and Subunits

Ribosome Preparation	Incorporation
	%
S. cerevesiae	
80s ribosome	100
60s subunit	5.2
40s subunit	5.0
60s and 40s	32.3
S. fragilis	
80s ribosome	100
60s subunit	5.2
40s subunit	4.8
60s and 40s	29.1

Preparation of 80s ribosomes and the isolation of 60s and 40s subunits are described in the legend to Figure 2. One OD_{260} unit of reconstituted 80s ribosomes, representing a 2:1 proportion of 60s and 40s subunits, was added in each assay. Incubation conditions are described in the legend to Figure 1. The original S. cerevesiae and S. fragilis 80s ribosomes incorporated 1000-3000 cpm/mg ribosomes. The data shown represent an average of 6 separate experiments.

Table II

Effect of Cycloheximide on Polyphenylalanine Synthesis Catalyzed by Reconstituted Ribosomes

Source of Subunit		Concentration of Cycloheximide Required for 50% Inhibition
60s	40s	M
S. fragilis	S. fragilis	$> 10^{-3}$
S. fragilis	S. cerevesiae	$> 10^{-3}$
S. cerevesiae	S. cerevesiae	5×10^{-5}
S. cerevesiae	S. fragilis	5×10^{-5}

Incubation conditions and composition of the reaction mixtures are described in the legends to Figure 1 and Table 1. 0.67 OD_{260} units of 60s and 0.33 OD_{260} units of 40s particles were used in each assay. A series of concentrations of cycloheximide were tested and the concentration required for 50% inhibition was determined as described under Materials and Methods. The data shown is representative of 7 separate experiments.

Table III

Effect of Inhibitors of the Transfer Reaction on Polyphenylalanine Synthesis by S. fragilis Ribosomes

Inhibitor	Concentration	Incorporation	Inhibition
	M	cpm/mg ribosomes	%
Streptovitacin A	10^{-4} M	1672	0
Emetine	10^{-4} M	1660	0
Cycloheximide	10^{-4} M	1650	0
Streptimidone	10^{-4} M	1132	25
	10^{-5} M	1600	5
Acetoxycycloheximide	10^{-4} M	1248	25
	10^{-5} M	1546	7
Tubulosine	10^{-4} M	1217	28
	10^{-5} M	1581	6
Anisomycin	10^{-4} M	300	82
	10^{-5} M	515	69

Assay conditions are the same as those described in the legend to Figure 1 except that 4.0 OD_{260} units of ribosomes and 56 ug of ^{14}C-phenylalanyl-tRNA (4,400) cpm were used in each assay.

ing dialysis, all of the activity in the original ribosomes was recovered. Separation of the subunits by sucrose density gradient centrifugation followed by reconstitution resulted in the recovery of approximately 30% of the original activity (Table I).

Effect of cycloheximide on reconstituted "hybrid" ribosomes: 60s and 40s ribosomal subunits, purified from S. fragilis and S. cerevesiae, were found to have little transfer activity when assayed individually (Table I). The subunits were then recombined in the ratio of 2:1 based on their absorbancy at 260 mμ and tested for their ability to incorporate ^{14}C-phenylalanine from ^{14}C-phenylalanyl-tRNA into polyphenylalanine. It can be seen from the data in Table II that the combination of the 60s subunit from S. fragilis

and the 40s subunit from S. cerevesiae was as resistant to cycloheximide as the homologous reconstituted system from S. fragilis. The corresponding "hybrid" particle, formed from the 40s subunit of S. fragilis and the 60s subunit of S. cerevesiae, was sensitive to the effects of the antibiotic.

Sensitivity of S. fragilis ribosomes to other inhibitors of the transfer reaction: Table III compares the inhibition of polyphenylalanine synthesis on S. fragilis ribosomes by anisomycin, emetine, tubulosine and several glutarimide antibiotics related to cycloheximide. Emetine and streptovitacin A resemble cycloheximide in their lack of effect; streptimidone, acetoxycycloheximide, anisomycin and tubulosine inhibit peptide synthesis at a concentration of 10^{-4} M.

DISCUSSION

The present studies confirm earlier reports of the resistance of ribosomal preparations of S. fragilis to cycloheximide (Siegel and Sisler, 1965) and the sensitivity of comparable extracts of S. cerevesiae (Cooper et al., 1967). The experiments described indicate that resistance of S. fragilis to cycloheximide is associated with the 60s ribosomal subunit. This resistance could result from differences in (1) ribosomal protein, as appears to be the case with streptomycin-resistant strains of E. coli (Leboy et al., 1964) (2) ribosomal conformation or (3) binding affinities of enzymes or co-factors for the ribosomes. Peptide synthesis on reconstituted ribosomes of S. fragilis was not stimulated by the addition of supernatant enzymes, indicating that the necessary enzymes and co-factors for polyphenylalanine synthesis are tightly associated or possibly an integral part of the ribosomal subunits. As the precise mode and site of cycloheximide action have not been established, it is not

possible to distinguish between the foregoing alternatives on the basis of present information.

It is generally assumed that the family of glutarimide antibiotics, including acetoxycycloheximide, streptomycin A and streptimidone, act on protein synthesis in the same manner as cycloheximide (Sisler and Siegel, 1967). A structural analogy has also been made between emetine, anisomycin, tubulosine and the glutarimide compounds based on configurational similarities and their activity as inhibitors of the transfer reaction (Grollman, 1966, 1967a, 1967b). The different sensitivity of S. fragilis ribosomes towards these agents indicate an apparent functional difference between these compounds. A comparison of the structural and conformational features of these inhibitors suggests that there may be two functional sites on those molecules. The functional groups which interacts with the Saccharomyces ribosome may not be identical with those which are responsible for the inhibition of protein biosynthesis.

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