INFLUENCE OF A PROTEIN SYNTHESIS INHIBITOR FROM BARLEY SEEDS UPON DIFFERENT STEPS OF ANIMAL CELL-FREE PROTEIN SYNTHESIS

by

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The mechanism of action on animal cell-free protein synthesis by the translation inhibitor II from barley seeds has been studied using the rabbit reticulocyte lysate protein synthesis system. No influence was detected on the ribosomal binding of initiator-tRNA, Met-tRNA_i, in the presence of barley hordein mRNA, or on the aminoacyl-tRNA synthetase catalyzed formation of aminoacyl-tRNA, suggesting the inhibitor to be without effect both on the protein synthesis initiation and on the supply of aminoacyl-tRNAs. Inhibition of reactions in the elongation cycle was examined by measurement of the transfer of the nascent peptide from peptidyl-tRNA to puromycin. The observed lack of influence on the puromycin reaction implies that the barley inhibitor neither blocked the peptidyltransferase nor the translocation activities. In addition, the high amount of Phe-tRNA bound to ribosomes in the presence of inhibitor and poly(U) suggested the aminoacyl-tRNA binding step to be unaffected by the inhibitor. These findings are compatible with inhibition of a reaction in the elongation cycle subsequent to the aminoacyl-tRNA binding but preceding the translocation.

1. INTRODUCTION

Three closely related inhibitors of animal cell-free protein synthesis have been purified from barley seeds (1). The amino acid sequence of the predominant form, barley translation inhibitor II, has recently been determined (2)

and found to be homologous with the A-chain of ricin from castor bean (12). Related proteins have been isolated from a wide variety of plants (3, 23, 24, 27) and extensive studies of structure and function have been performed for pokeweed antiviral proteins (PAP) and ricin (4, 5, 7, 10-13,

Abbreviations: EF = elongation factor; ID₅₀ = inhibitor concentration causing 50% inhibition of the in vitro translation; NEM = N-ethylmaleimide; PAP = pokeweed antiviral protein; poly(U) = polyuridylic acid; Tricine = N-[(2-hydroxy-1,1-bis (hydroxymethyl)ethyl)glycine; Tris = 2-amino-2-hydroxymethylpropane-1,3-diol.

21, 24-26). The barley proteins are less effective inhibitors than the ricin A-chain or PAP (1, 3, 23). The target reaction has only been identified for a few of this class of protein synthesis inhibitors. Therefore, in the present study we investigated the mode of action of the barley inhibitor II by testing for its effects on individual reactions of the *in vitro* translation, i.e. Met-tRNA_f binding in the initiation phase and peptidyltransferase reaction, translocation and ribosomal binding of aminoacyl-tRNA of the elongation step, using rabbit reticulocyte lysates.

2. MATERIALS AND METHODS

2.1. Materials

Barley translation inhibitor II (1) and barley hordein mRNA (6, 17) were prepared as described. An aminoacyl-tRNA synthetase preparation from stroma of barley plastids (19) was generously supplied by Dr. C. GAMINI KANNANGARA, Department of Physiology, Carlsberg Laboratory.

mRNA-dependent rabbit reticulocyte lysate (both depleted and not depleted with respect to endogenous amino acids, ribosome concentration approx. $270 \text{ pmol} \times \text{ml}^{-1}$ (22)) was from Amersham International, Amersham, U.K.; L-[³⁵S]-methionine, L-[¹⁴C]- and L-[³H]-phenylalanine from New England Nuclear Chemicals, Dreieich, F.R.G.; tRNA of calf liver from Boehringer Mannheim, Mannheim, F.R.G.; tRNA^{Phe} of brewers yeast, RNA of *Torula* yeast, poly(U), anisomycin, cycloheximide and *N*-ethylmaleimide from Sigma Chemical Co., St. Louis, MO; aurintricarboxylic acid (ammonium salt) and cetyltrimethylammonium bromide from E. Merck, Darmstadt, F.R.G.; glass fiber filter (GF/C) from Whatman, Maidstone, U.K.; nitrocellulose filter (HAWP) from Millipore, Molsheim, France. All other chemicals were of analytical grade.

2.2. Methods

2.2.1. Binding of initiator-tRNA to ribosomes

Amino acid-depleted rabbit reticulocyte lysate (15 μl) was preincubated in the presence or absence of various inhibitors (18) of protein synthesis at 30 °C for 15 min. The lysate was then incubated in a 20 μl -reaction mixture containing

0.44 μM -L-(35_s)-methionine (10 μCi) and barley hordein mRNA ($A_{260} = 11.0$, 2 μl) at 30 °C for 30 min. The reaction was stopped by adding 1 ml of ice-cold 50 mM-Tris-HCl pH 7.4 containing 60 mM-KCl and 6 mM-MgCl₂ (10). The mixture was then filtered on a nitrocellulose filter and after ten washings with 1 ml portions of the ice-cold buffer, the radioactivity of the retained ribosomes was measured.

2.2.2. Met- and Phe-tRNA synthesis

tRNA of calf liver (40 μg) and aminoacyl-tRNA synthetase (100 μg) were incubated, in the presence or absence of translation inhibitor, in 240 μl of 77 mM-Tricine pH 6.8 containing 2.8 mM-MgCl₂ at 30 °C for 15 min. Then 0.01 μM -L-(35_s)-methionine (3 μCi) and 1 mM-ATP were added and the reaction mixture was incubated at 30 °C for 30 min. Essentially the same procedure was used with tRNA^{Phe} (12 μg) and 0.58 μM -L-[³H]-phenylalanine (12 μCi). The reaction was stopped by adding 10 μl of 50% trichloroacetic acid. The mixture was filtered on a glass fiber filter and after washing with three 5 ml portions of cold 5% trichloroacetic acid, the radioactivity of the retained aminoacyl-tRNA was measured.

2.2.3. *In vitro* translation systems

Rabbit reticulocyte lysate (55 μl) was incubated in a 70 μl reaction mixture containing 0.63 μM -L-(35_s)-methionine (50 μCi) and barley hordein mRNA ($A_{260} = 11.0$, 5 μl) at 30 °C. For poly(U) directed polyphenylalanine synthesis, amino acid depleted rabbit reticulocyte lysate (15 μl) was incubated in a 30 μl reaction mixture containing 119 μM -L-[¹⁴C]-phenylalanine (1.6 μCi), 33 μM -non-radioactive amino acids (omitting L-phenylalanine), 67 mM-potassium acetate, 3.3 mM-magnesium acetate, 1.7 mM-ATP, 10 μg of t-RNA^{Phe}, aminoacyl-tRNA synthetase (100 μg) and 20 μg of poly(U) at 30 °C for 30 min. The incorporated radioactivity was measured on 10 μl aliquots as described in 2.2.4.

2.2.4. Analysis of the puromycin reaction

The amount of peptidyl-tRNA was measured essentially as reported (16). Puromycin was

added to the translation mixtures as described in Figure 1. In order to precipitate peptidyl-tRNA, aliquots of translation mixture (5 μ l, see section 2.2.3) were added 1 ml of 2% cetyltrimethylammonium bromide followed by 1 ml of 0.5 M-sodium acetate pH 5.4 containing 0.5 mg $\cdot$ ml⁻¹ of yeast RNA as carrier. After 30 min, the precipitates were collected on glass fiber filters and washed thoroughly with water. The filters were then boiled in 10% trichloroacetic acid for 10 min to remove aminoacyl-tRNA, washed with three 5 ml portions of 5% cold trichloroacetic acid and dried. The radioactivity retained on the filters was measured.

2.2.5. Inhibition of translation by *N*-ethylmaleimide

Amino acid depleted rabbit reticulocyte lysate was treated with 10 mM-*N*-ethylmaleimide at 0 °C for 10 min (15). Remaining *N*-ethylmaleimide was reacted with 20 mM-mercaptoethanol and the lysate was checked for the loss of ability to synthesize polyphenylalanine using the conditions given in 2.2.3.

2.2.6. *Phe*-tRNA binding to ribosomes

Amino acid depleted, NEM-treated rabbit reticulocyte lysate (30 μ l) (see 2.2.5) was preincubated with translation inhibitor (20 ng) at 30 °C for 15 min and subsequently incubated at 30 °C for 30 min under the conditions given for poly(U) directed polyphenylalanine synthesis (see 2.2.3). The reaction was stopped and the radioactivity associated to ribosomes was measured (see 2.2.1).

2.2.7. Analytical procedures

Concentrations of barley translation inhibitor II in stock solutions were determined by amino acid analysis (1). Radioactivity was measured in a Beckman LS 3155T liquid scintillation counter.

3. RESULTS AND DISCUSSION

3.1. Met-tRNA_i ribosomal binding in the presence of inhibitor

Protein synthesis in eukaryotes is initiated by the binding of Met-tRNA_i to the 40S ribosomal subunit, followed by the association with mRNA and the 60S ribosomal subunit. The effect of the translation inhibitor on the binding of the initiator-tRNA to ribosomes was examined using amino acid depleted rabbit reticulocyte lysate in the presence of radioactive methionine. Like ricin and PAP (3) the barley inhibitor did not reduce the binding of Met-tRNA_i (Table I), in contrast to the inhibitor of protein synthesis initiation, aurintricarboxylic acid (18), employed as a control (Table I). Furthermore, to eliminate the possibility that the inhibition of translation could stem from lack of activated amino acids, aminoacyl-tRNA synthetase catalyzed formation of Met- and Phe-tRNA was examined. Apparently the yields of aminoacyl-tRNAs were unaffected by the presence of inhibitor (Table II), thus the supply of aminoacyl-tRNAs was assumed to be sufficient for efficient translation to take place.

Table I. The effect of the barley translation inhibitor on the initiation step of protein synthesis.

Inhibitor added	Binding of methionyl-tRNA	
	Radioactivity incorporated (cpm $\cdot$ 10 ⁻³)	% control
Translation inhibitor (16 nM) ^{a)}	217	107
Aurintricarboxylic acid (10 mM)	76	37
None	203	100

a) Approx. 20 times as high a concentration as ID₅₀ (1). See 2.2.1 for further details.

Table II. Synthesis of aminoacyl-tRNA in the presence of barley translation inhibitor.

Translation inhibitor (nM)	Radioactivity incorporated (cpm · 10 ⁻³)	
	Met-tRNA	Phe-tRNA
33 ^{a)}	120	—
330 ^{b)}	—	7
None	120	7

a) Approx. 40 times the ID₅₀ for in vitro translation of barley hordein mRNA (1).

b) Approx. 33 times the ID₅₀ for the poly(U) directed polyphenylalanine synthesis.

See 2.2.2 for further details.

3.2. Peptidyl transfer to puromycin after inhibitor treatment

Puromycin, an analogue of the 3'-end of aminoacyl-tRNA, binds at or near the ribosomal A-site and accepts the peptidyl moiety of peptidyl-tRNA located in the P-site to form peptidyl-puromycin which is subsequently released from the ribosome (28). This reaction is specific for P-site located peptidyl-tRNA at normal salt concentrations and has previously been utilized to classify antibiotics with respect to target reaction in the elongation cycle. In the present study the puromycin reaction was used to test if the barley inhibitor interfered with the translocation or the peptidyltransferase reactions (Fi-

Figure 1. The effect of the translation inhibitor on the puromycin reaction. In vitro translation reaction mixture containing barley hordein mRNA (70 μ l) (see 2.2.3) was incubated for ten minutes. Then 0.2 mM-puromycin (P₀) was added to a 15 μ l portion of the reaction mixture and a) 3.6 μ M-barley translation inhibitor (T), b) 1 mM-anisomycin (A) or c) 0.9 mM-cycloheximide (C) was added to a portion 40 μ l of the same reaction mixture. After 15 min of incubation, 0.2 mM-puromycin (P) was added to a 20 μ l portions of the reaction mixtures a-c. Aliquots 5 μ l were removed with the indicated time intervals and the content of peptidyl-tRNA was measured as described in section 2.2.4. The symbols correspond to the noninhibited rabbit reticulocyte lysate system (○), the system inhibited by the barley translation inhibitor, anisomycin or cycloheximide (●), the puromycin-treated noninhibited system (△) and the puromycin-treated system inhibited by either of the three inhibitors (▲).

Table III. Inhibition of polyphenylalanine synthesis by NEM.

Inhibitor added	Polyphenylalanine synthesis	
	Phenylalanine incorporated	% inhibition
	approx. pmol	
None	43	0
N-ethylmaleimide	3	93

Rabbit reticulocyte lysate (15 μ l, 4 pmol ribosomes) depleted of all amino acids was treated with NEM (see 2.2.5). Before and after this inactivation the polyphenylalanine synthesis of the lysate was assayed (see 2.2.3).

figure 1a). In control experiments we confirmed the function of two well-characterized antibiotics, cycloheximide and anisomycin (16, 18). In the presence of anisomycin, the puromycin reaction was completely inhibited due to a blocked peptidyltransferase reaction (Figure 1b). The translocation arrest caused by cycloheximide resulted in a partial inhibition of the puromycin reaction due to inability of A-site located peptidyl-tRNA to be transferred to the puromycin sensitive P-site (Figure 1c). As seen in Figure 1a the barley inhibitor was without effect on the puromycin reaction, even at a concentration of 4500 times the ID_{50} , which suggests that it blocks neither the peptidyltransferase reaction nor the translocation. In contrast, ricin A-chain and PAP are both thought to inhibit the translocation (13, 26). This behaviour of the barley inhibitor resembles that of the mold translation inhibitor α -sarcin, but α -sarcin specifically cleaves a single phosphodiester bond

in 28S ribosomal RNA (9), while the barley inhibitor has no ribonuclease activity (1). It is obvious, therefore, that the mode of action of the barley inhibitor differs from those previously characterized for plant and mold translation inhibitors.

3.3. Ribosomal binding of Phe-tRNA in the presence of inhibitor

In order to study the isolated event of poly(U) directed ribosomal binding of Phe-tRNA in the reticulocyte system, we destroyed the activity of EF-2 by NEM-treatment (15). The extent of Phe-tRNA binding at the ribosomal A-site was then measured in the presence of barley translation inhibitor employing the NEM-treated reticulocyte lysate. Although the NEM-treatment efficiently prevented incorporation of labelled phenylalanine into the hot-acid insoluble material (Table III), a small fraction of the EF-2

Table IV. The effect of the translation inhibitor on the binding of Phe-tRNA to NEM-treated ribosomes.

Inhibitor added	Binding of Phe-tRNA	
	Phenylalanine incorporated	% control
	approx. pmol	
None	63	100
Translation inhibitor	3	5

NEM-inhibited lysate as described in Table III (30 μ l, 8 pmol ribosomes) was preincubated with 20 ng of the translation inhibitor at 30 °C for 15 min, and then further incubated at 30 °C for 30 min under the condition employed for translation of poly(U) (see 2.2.3). The reaction was stopped (see 2.2.1) and the radioactivity associated with the ribosomes retained on the filter was measured.

remained active allowing the synthesis of short oligopeptides on the poly(U) programmed ribosomes (Table IV). Preincubation of the NEM-treated lysates with 21 nM translation inhibitor (ID_{50} for polyphenylalanine synthesis is 10 nM) reduced the amount of labelled phenylalanine associated with ribosomes to a level corresponding to a molar ratio of 0.4 Phe-tRNA to ribosome (Table IV). A similar value and lower values were previously reported for Phe-tRNA binding to untreated, poly(U) programmed ribosomes (8, 14, 20). Thus, the inhibitor seemed not to influence the Phe-tRNA binding, but stopped the residual translation in the NEM-treated lysate, as expected.

4. CONCLUSION

The present study concerns the mode of inhibition of *in vitro* animal protein synthesis by the translation inhibitor II from barley seeds. This inhibitor has apparently no effect on the initiation phase and the aminoacyl-tRNA binding, peptidisation and translocation of the elongation phase, thus the mode of action is indirectly demonstrated to differ from that of the other structurally related translation inhibitors. A target reaction of the barley translation inhibitor still remains to be identified, but it seems possible that the translation is arrested at a partial reaction of the elongation cycle between the aminoacyl-tRNA binding and the translocation. Due to the complexity of the reticulocyte lysate protein synthesis system, studies of an individual reaction in the elongation impose great experimental difficulties, and it was decided, therefore, to investigate further the mode of action of the barley inhibitor in reconstituted systems for protein synthesis (22).

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