INHIBITION OF PHENYLALANINE tRNA SYNTHETASE FROM *BACILLUS SUBTILIS* BY OCHRATOXIN A

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Received 5 September 1977

1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced by some strains of *Aspergillus ochraceus, Penicillium viridicatum* and some other *Aspergillus* species [1].

It is composed of a phenylalanine moiety bound by a α-amide-binding to an isocoumarin moiety. The isocoumarin system is chlorinated in the 5′ position.

The molds, producing this mycotoxin, are found ubiquitous; contaminations and production of the mycotoxin were reported especially in cereals [2].

In animals OTA produces particularly liver and kidney damages [1]. OTA also has antibiotic properties. It inhibits growth of gram-positive bacteria but no growth inhibition was found with gram-negative species [3]. In *Streptococcus faecalis* OTA inhibits RNA and protein synthesis to the same extent but not DNA synthesis. Attempts to inhibit an in vitro protein synthesis system from *E. coli* with OTA failed. Assays in which growing protoplasts of *E. coli* were treated with OTA gave only a very slight inhibition with concentrations of several 100 μg/ml, i.e., at a 100–1000-fold higher concentration than with gram-positive bacteria.

In *B. subtilis* the inhibition in vivo of protein synthesis by OTA is stronger than that of RNA synthesis. An investigation of the nucleotide pools showed a decrease in ATP and GTP pools and an increase in the MS-nucleotides ppGpp and pppGpp by about 4-fold [4]. As such a result is generally interpreted as meaning the binding to the ribosomes of an uncharged tRNA [5], we investigated the effect of OTA on the acylation of tRNA by *B. subtilis* tRNA synthetase.

2. Materials and methods

Ochratoxin A was isolated and purified from wheat kernels infected by *Aspergillus ochraceus* as described [3].

Bacillus subtilis 168 I^- (requiring tryptophan or indole) was grown in a New Brunswick microferm fermentor at maximal aeration and agitation. The growth medium contained per 10 liter: peptone, 150 g; yeast extract, 400 g; sodium glutamate, 10 g; $MgSO_4$, 2.0 g; $K_2HPO_4 \times 3\ H_2O$, 140 g; KH_2PO_4, 60.0 g; sodium citrate, 1.0 g; $MnCl_2 \times 4\ H_2O$, 13 mg; $(NH_4)_2SO_4$, 2.0 g; glucose, 50.0 g.

The cells were chilled and harvested in the late exponential growth phase, and 20.5 g cells (wet wt) per liter medium were obtained.

The phenylalanine tRNA synthetase was prepared according to the following procedure: 25 g cells (wet) were suspended in a prewarmed Tris/HCl buffer, pH 8.0 containing 0.15 M NaCl, 1 mM EDTA, and 20% sucrose. Per gram of cells 40 μg lysozyme were added and the suspension was incubated at 37°C for 1 h. Then the suspension was chilled to 0°C and sonified 3 × 15 s with a Bronson sonifier at the setting of 70 W. Before sonifying 0.002% of phenylmethylsulfonylfluoride were added to the suspension.

The sonified suspension was centrifuged at 20 000 × *g* for 10 min at 4°C. To the supernatant 10 μg/ml DNAase were added before centrifugation at 100 000 × *g* for 2 h. The supernatant from this centrifugation was precipitated by 55 g/100 ml $(NH_4)_2SO_4$ and the precipitate collected by centrifugation at 100 000 × *g* for 10 min.

The pellet was dissolved in a 20 mM potassium phosphate buffer, pH 7.5, which also contained protease inhibitor (1 ml 2% solution of phenylmethylsulfonylfluoride in 1 liter buffer) and was dialyzed for 12 h against the same buffer.

This extract was added onto a DEAE-cellulose column and was eluted by a linear gradient of 0.020–0.250 M potassium phosphate (300 ml 0.020 M K-phosphate, pH 7.5, and 300 ml 0.250 M K-phosphate, pH 6.3). Thereafter the elution with 0.250 M K-phosphate buffer, pH 6.3 was continued. The transmission at 280 nm of the eluate was monitored with a UV-monitor (Uvicord, LKB), and fractions of 10 ml were collected.

The phenylalanine and valine t-RNA synthetase activity and the activity toward an amino [^{14}C]acid mixture containing 15 amino acids in relative proportions as found in a typical algal hydrolysate (NEN chemicals), was checked and was found to be distributed as shown in fig.1. The fractions marked by the arrows were utilized for the respective assays. The enzyme preparations contained 0.66 mg/ml protein in fraction 58, 0.44 mg/ml in fraction 69 and 1.16 mg/ml in fraction 42. For the preparation of *B. subtilis* t-RNA the method of Vold [6] was followed; the *E. coli* tRNA was purchased from General Biochemicals, Chagrin Falls, OH. For the concentration of 1 mg/ml an absorption at 260 nm of 24 was assumed.

The amino acylation mixture contained Tris/HCl, pH 7.0, 4 mM; $MgCl_2$, 0.01 M; KCl, 0.01 M; glutathion, 0.01 M; ATP, 0.01 M; synthetase 10 μl; variable amounts of amino acids as indicated; 0.100 mg tRNA/100 μl for *E. coli* and 0.046 mg tRNA/100 μl for *B. subtilis*.

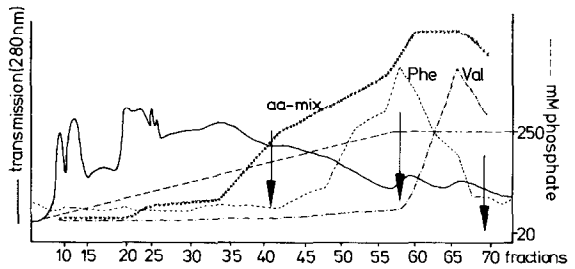

Fig.1. Elution diagram of a *B. subtilis* extract from DEAE-cellulose. The tRNA synthetase activites are shown for phenylalanine and valine. The curve marked 'aa-mix' shows the activity against an amino [^{14}C]acid mixture containing 15 amino acids. The arrows mark the fractions utilized.

The difference in the concentrations between *E. coli* tRNA and *B. subtilis* tRNA was not found to be responsible for a better amino acylation in the heterologous system. In equal concentrations of *E. coli* tRNA or *B. subtilis* tRNA in the reaction mixture the reaction rate in the heterologous system was distinctly higher and the levels of amino acylation were significantly higher.

A time dependence curve was made which showed that the amino acylation reaction for both tRNAs was linear at least for the first 7 min after starting the reaction. Therefore, the kinetic measurements were all carried out at 7 min after starting the reaction. The amino acylated tRNA was precipitated with 5% trichloroacetic acid and the radioactivity was counted on membrane filters (Sartorius) in a liquid scintillation counter (Packard).

The standard error for this procedure was estimated to be better than ±5%.

The radioactive materials were a generous gift from Dr P. Fromageot, CEA, Saclay, France.

3. Results

From preliminary experiments we knew that the acylation of phenylalanine tRNA from *E. coli* by synthetase from *B. subtilis* is inhibited by OTA [4]. Data from the literature [7] and own assays showed that it is not likely that OTA binds to nucleic acids, and therefore, should not have an effect on tRNA.

Table 1 shows the results of an assay in which a combination of tRNAs and synthetases from both organisms was used.

Only the activity of the phenylalanine tRNA synthetase from *B. subtilis* could be inhibited. The origin of the tRNAs applied in the assays was not important. The acylation was inhibited in the heterologous system as well as in the homologous if the t-RNA synthetase was derived from *B. subtilis*.

The *B. subtilis* synthetase was used in all the further experiments in order to investigate the homologous and heterologous system, respectively.

Figure 2 shows the dependence of the inhibition on the OTA concentration. In the homologous system (fig.2b) no further increase in inhibition was

Table 1
Effect of ochratoxin A on acylation of phenylalanine t-RNA by synthetase from *E. coli* and *B. subtilis*

Synthetase	tRNA	OTA concentration (μg/ml)	% Inhibition
		0	0
		5	0
E. coli	*E. coli*	10	0
		15	0
		0	0
		5	0
E. coli	*B. subtilis*	10	0
		15	0
		0	0
		5	14
B. subtilis	*E. coli*	10	50
		15	55
		0	0
		5	39
B. subtilis	*B. subtilis*	10	65
		15	67

observed above a concentration of about 10 μg/ml, while in the heterologous system (fig.2a) a certain decrease in activity was found up to 100 μg/ml.

A dependence of the level of amino acylation on the ratio of synthetase to tRNA concentration existed in the homologous system above an enzyme concentration of 9.9 μg/ml, the tRNA concentration being kept constant. The labelling decreased by about 20% when the enzyme concentration was raised from 9.9–16.5 μg/100 μl. This fact is reflected in fig.2b. An increase in the enzyme concentration from 9.9–13.2 μg/

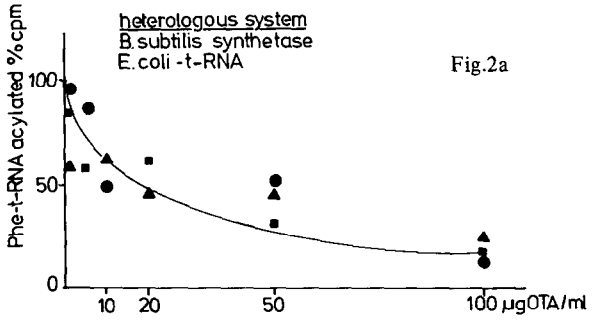

Fig.2a

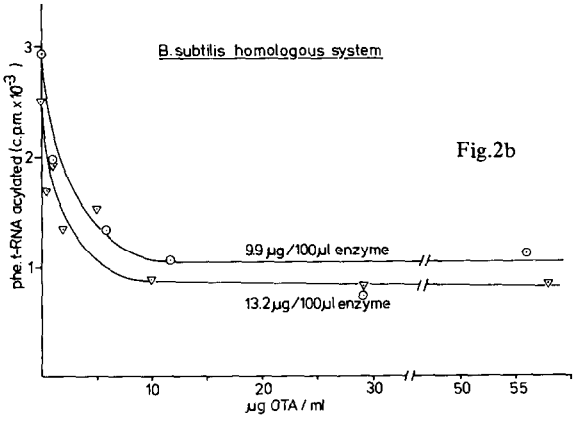

Fig.2b

Fig.2. Inhibition of phenylalanine acylation by ochratoxin. (a) Amino acylation, at pH 7.0, with synthetase of fraction 58, and different concentrations of enzyme, heterologous system with *E. coli* t-RNA. (●–●) 0.66 μg; (■–■) 3.3 μg; (▲–▲) 6.6 μg enzyme/100 μl acylation mixture. The data are taken from 6 independent experiments. The line marks a crude estimation of the concentration dependence. (b) Inhibition of the phenylalanine acylation in the homologous system with two enzyme concentrations as indicated.

Fig.3a

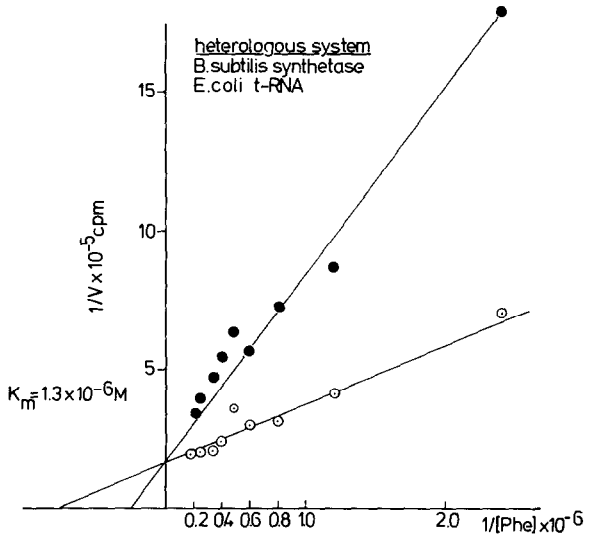

Fig.3b

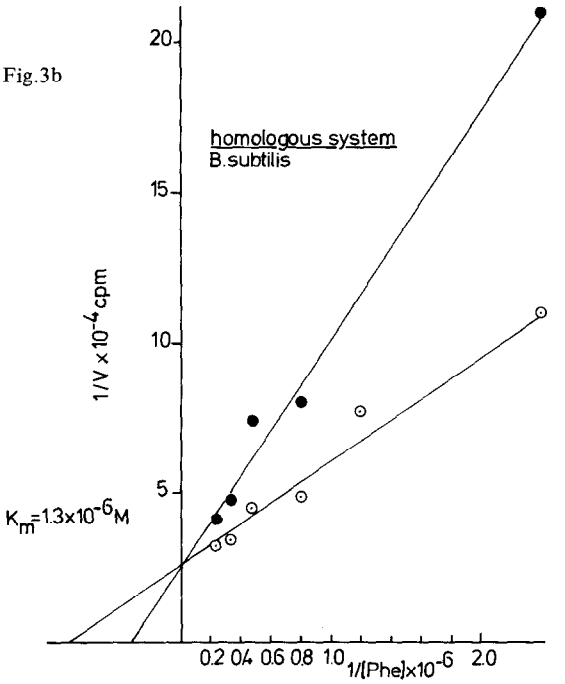

Fig.3. Lineweaver-Burk plots of the inhibition of phenylalanine acylation by ochratoxin. The measurements were made 7 min after starting the reaction. Fraction 58 was utilized. (a) Heterologous system with *E. coli* tRNA. (⊙—⊙) control; (•—•) in presence of 10 μg/ml of ochratoxin. Data represent a single determination out of a series of three independent experiments. The lines were fitted according to the most probable inclinations in respect to the data from all three experiments. (b) Homologous system. (⊙—⊙) control; (•—•) in presence 5μg/ml of ochratoxin.

100 μl caused a decrease of the amino acylation by about 15%.

The type of inhibition which OTA exerts on the phenylalanine tRNA synthetase of *B. subtilis* is illustrated in fig.3b. The homologous system as well as the heterologous is inhibited competitively by OTA in the concentration range of phenylalanine as indicated.

The K_m is the same for both systems; however, the velocity of the reaction is higher in the heterologous system (fig.3a), which is in agreement with the observation that the heterologous system is better charging than the homologous (fig.3b). In fig.4 the K_i-values are shown. The K_i-values are about 10-times higher in the heterologous system (fig.4a) than they are in the homologous, and thus are about 25 times higher than the K_m. In the homologous system, however, (fig.4b) the K_i is only about 2 times higher than the K_m.

In order to obtain an idea on the specificity of the inhibition of amino acylation we used tRNA synthetase fractions (fig.1) which did not contain phenylalanine charging activities. With an amino [^{14}C]acid mixture a maximal inhibition in the homologous system of about 20% was observed in both fractions used.

In the heterologous system the same applied for fraction 42, which might still contain low levels of phenylalanine synthetase activity. In fraction 69 no inhibition was observed but rather a marginal stimulation.

The same was the case for the acylation of [^{14}C]-valine. As the valine synthetase activity in the homologous system was rather low the value of 29% inhibition with 7 μg/ml is probably erroneous.

Thus, although we have not assayed all 20 amino acid tRNA synthetases separately, it seems quite evident that OTA inhibits rather specifically phenyl-

Fig.4a

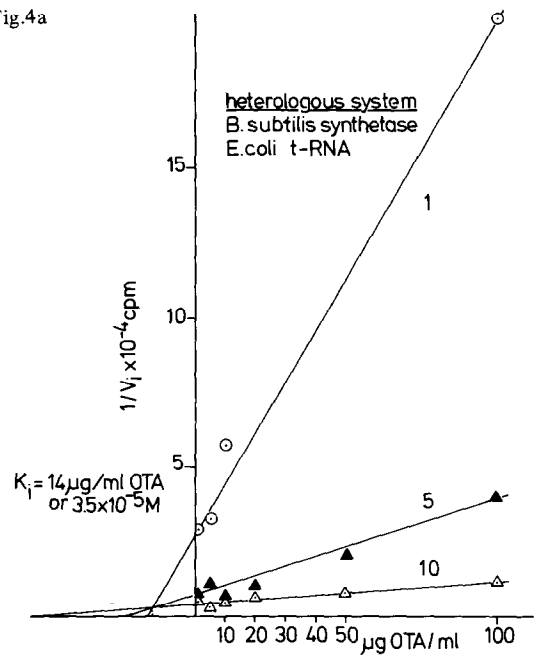

Fig.4b

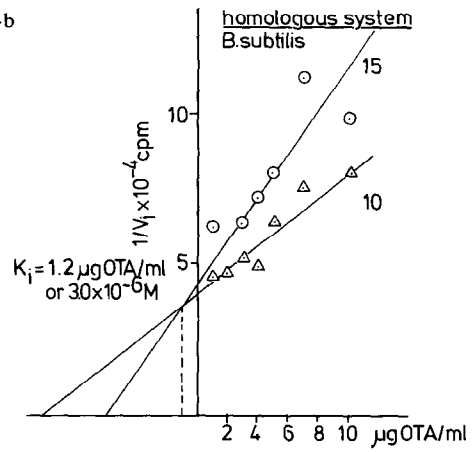

Fig.4. Dixon plots of the inhibition of phenylalanine acylation by ochratoxin. The measurements were made 7 min after starting the reaction. Fraction 58 was used. The numbers on the respective curves designate: 1 = 0.66 µg (1 µl), 5 = 3.3 µg (5 µl), 10 = 6.6 µg (10 µl), 15 = 9.9 µg (15 µl) enzyme/100 µl acylation mixture. (a) Heterologous system with *E. coli* t-RNA. (b) Homologous system.

Table 2
Specificity of inhibition

Amino [^{14}C]acid mixture used for charging of t-RNA

Fraction[a]	Concentration OTA (µg/ml)	Homologous cpm	% inhib.	Concentration OTA (µg/ml)	Heterologous cpm	% inhib.
	0	2212	0	0	5696	0
42	5	1862	16	5.8	4327	24
	10	1817	18	11.6	4397	23
	0	2582	0	0	6329	0
69	5	1992	23	5.8	8628	0
	10	2088	19	11.6	6776	0

[^{14}C]Valine and t-RNA synthetase fraction[a] 69 used for charging

Concentration OTA (µg/ml)	Homologous cpm	% inhib.	Concentration OTA (µg/ml)	Heterologous cpm	% inhib.
0	322	0	0	11 973	0
1	325	0	1	11 257	6
2	305	5	5.8	11 919	0.5
3	262	18	11.6	12 982	0
5	265	18	23.6	12 922	0
7	229	29	46	12 418	0
10	271	16	115	12 563	0

[a] The numbers of the fractions are taken from fig.1

alanine tRNA synthetase of *Bacillus subtilis*. In an attempt in order to ascertain whether the higher activity of the tRNA synthetase for phenylalanine in the heterologous system is due to an unspecific charging, tyrosine, and valine were assayed with fraction 58, but no amino acylation of *E. coli* tRNA occured with these amino acids.

Another phenomenon was observed which might be interesting for the investigation of the mechanism of amino acylation. After several weeks of keeping the phenylalanine t-RNA synthetase in a frozen state the activity in the homologous system increased, and at the same time the extent of inhibition by OTA in both systems decreased and finally the reaction in the homologous system could not be inhibited anymore. The synthetase, however, still showed good transfer activity which still could be inhibited to a certain extent in the heterologous system.

4. Discussion

Protein synthesis has been shown to be inhibited in vivo by ochratoxin A in *Bacillus subtilis* [4] and *Streptococcus faecalis* [3]. The inhibition of RNA synthesis in these bacteria has to be regarded as a secondary regulation effect as it has been shown that in both bacterial species RNA synthesis is not inhibited in presence of chloramphenicol and ochratoxin. In *B. subtilis* the pools of both MS-nucleotides increase by about 4-fold [4] while the pools of ATP and GTP decrease. In *Streptococcus faecalis* the polysomes are stabilized in the first 6 min after addition of ochratoxin which would support the idea of an inhibition of elongation of the peptide synthesis. The increase of the MS-nucleotides in *B. subtilis* on the other hand, is regarded as a sign that uncharged tRNA might be bound to the ribosomes [5]. Therefore, we decided to investigate this reaction more closely.

Preliminary results with an *E. coli* system had shown no inhibition of the amino acylation of several amino acids nor an inhibition of a poly(U)-dependent cell-free protein synthesis system.

As it is well known that many antibiotics can not penetrate the outer membrane of gram-negative bacteria, we checked the incorporation of radioactive amino acids or of uracil into acid precipitable material of growing *E. coli* protoplasts. But only at 100–1000-fold higher concentrations as necessary to inhibit growth of *B. subtilis* a relatively small inhibition of protein- and RNA synthesis could be observed. Thus, the difference in the sensitivity of gram-positive and gram-negative bacteria to ochratoxin did not seem to be due to a penetration problem. On the other hand, it is believed that at least in *E. coli* [8] the amino group of an amino acid analog should be unsubstituted if the compound should act as inhibitor of the amino acylation reactions. This applies also to tyrosine tRNA synthetase of *B. subtilis* [9]. Nevertheless, our results show clearly that it is the *B. subtilis* tRNA synthetase for phenylalanine which is inhibited while for *E. coli* the considerations mentioned may be valid. Thus, our results support the notion that ochratoxin is a competitive inhibitor of the overall reaction of phenylalanine acylation in *B. subtilis*, i.e., acts as analog of phenylalanine. Whether it is the amino acid activation reaction or the transfer reaction which is inhibited has not yet been investigated.

An investigation of in vivo amino acylation of the tRNA in *B. subtilis* by the periodate oxidation method [10] always showed the lowest levels for phenylalanyl-tRNA after ochratoxin treatment of the cells. But also the control cultures showed lower levels as expected. Also the valyl-tRNA which was investigated in comparison showed rather low levels of amino acylation. Recently a paper of Tockman and Vold [11] appeared in which was shown that the periodate oxidation method in *B. subtilis* can lead to destruction of several amino acylated tRNAs and that results obtained by this method have to be interpreted with caution. We, therefore, did not include these data in the paper despite the fact that they are rather supporting the notion that ochratoxin inhibits phenylalanine t-RNA acylation in vivo. The fact that an older enzyme preparation changed its properties concerning the activity and the sensitivity to ochratoxin could give rise to speculations on a change of conformation of the enzyme which also could be connected with the fact that the heterologous system normally shows a higher activity than the homologous. But until now there are not enough data on this enzyme available to interpret these phenomena conclusively.

Acknowledgement

We wish to thank the Deutsche Forschungsgemeinschaft for supporting this project.

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