NOTE

SURVEY OF INHIBITORS IN DIFFERENT STEPS OF PROTEIN SYNTHESIS BY MAMMALIAN RIBOSOMES

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An increasing number of antibiotics has been reported in the last few years to be active on 80S type ribosomes[1,2,3,4]. However taking into account the scarcity of data concerning the site and mode of action of these antibiotics and the enormous heterogeneity of the biological sources of 80S type ribosomes in which they were tested we have considered of interest surveying a number of inhibitors in different steps of protein synthesis using in all cases the same mammalian cell-free system. We summarize in this contribution suitable methods to study the individual steps of protein synthesis by human tonsil ribosomes and present the results obtained in these systems in the presence of a number of protein synthesis inhibitors.

Materials and Methods

Human tonsil ribosomes, elongation factor 1 (EF 1) and elongation factor 2 (EF 2) were obtained and purified as previously described.[5,6,7,8] Purity of EF 2 after phosphocellulose chromatography[7] was approximately 50 %.[9]

^{14}C-Phe-tRNA was prepared from *Escherichia coli* B tRNA (Sigma) that was charged with ^{14}C-phenylalanine (513 mCi/mmol) (The Radiochemical Centre, Amersham, England) using a partially purified supernatant preparation of *E. coli*.[10] Ac-^{14}C-Phe-tRNA was prepared by acetylation of ^{14}C-Phe-tRNA (tRNA used for this purpose was from baker's yeast) as described by other workers[11] and then separated from deacilated tRNA by BD-cellulose chromatography following a method which basically is similar to others already described[12]. Ac-^{14}C-Phe-tRNA of specific activity 513 mCi/mmol was obtained.

The following buffers were used in the assays described below: Buffer A (20 mM Tris-HCl, pH 7.4, containing 60 mM KCl and 7 mM $MgCl_2$), buffer B (50 mM Tris-HCl, pH 7.4, containing 60 mM KCl, 11 mM $MgCl_2$ and 8 mM 2-mercaptoethanol), buffer C (20 mM Tris-HCl, pH 7.4, containing 60 mM KCl and 5 mM $MgCl_2$) and buffer D (20 mM Tris-HCl, pH 7.4, containing 60 mM KCl and 15 mM $MgCl_2$).

Enzymic binding of ^{14}C-Phe-tRNA to ribosomes was studied in buffer C following essentially methods already described using other mammalian systems.[13] In order to isolate, when required, the complex ribosome-^{14}C-Phe-tRNA (with ^{14}C-Phe-tRNA enzymically bound to the ribosomal A-site) the following components were incubated in buffer A for 30 minutes at 37°C in a final volume of 5 ml: 1 mg poly U, 250 pCi ^{14}C-Phe-tRNA, 3 mg protein EF 1 preparation, 1 μmol GTP and 3.5 mg human tonsil ribosomes. Complex formation was started by addition of GTP. At the end of the incubation period the incubation mixture was chilled on ice and a 50 μl sample was taken to estimate formation of the complex ribosome-^{14}C-Phe-tRNA by Millipore filtration.[13] Approximately 12 % of the total ribosomes in the incubation mixture were forming the complex. The total of the chilled mixture was taken and placed over 7 ml of a 10 % sucrose solution in buffer B in 12 ml tubes of the Spinco/Beckman ultracentrifuge and centrifugation was carried out for 3 hours at 105,000 *g* at 4°C. The pellet was carefully rinsed for three times, without removing the pellet, with buffer B and resuspended in 0.5 ml of the same buffer. Absorbancy at 260 nm and formation of the complex ribosome-^{14}C-Phe-tRNA was then estimated. The complex suspension was stored at 0°C and was stable at least for two weeks.

The ribosome-^{14}C-Phe-tRNA complex prepared as described above was used to study translocation. For this purpose 100 μl mix-

tures of buffer B were used containing 2.4 pmoles ribosome-^{14}C-Phe-tRNA complex, 70 mg EF 2 and 0.4 μmoles GTP. The reaction was initiated by addition of GTP and incubation carried out for 15 minutes at 37°C. To stop translocation the reaction mixture was chilled on ice at the end of the incubation period and placed over 12 ml of a 10 % sucrose solution in buffer B in tubes of the Spinco/Beckman ultracentrifuge and the complex sedimented by centrifugation for 3 hours at 105,000 g at 4°C. The pellet was resuspended in 100 μl buffer B at 24 pmoles/ml. The extent of the translocation reaction was estimated by the puromycin reaction with this resuspended pellet as described below: Some 20~30 % of the total ^{14}C-Phe-tRNA bound was translocated.

Reaction with puromycin of the ribosome-^{14}C-Phe-tRNA complex obtained after translocation as indicated above, was studied in 100 μl incubation mixtures in buffer B containing 0.7 pmoles of translocated ribosome-^{14}C-Phe-tRNA complex and 0.1 μmole puromycin. Incubation was carried out for 20 minutes at 37C. Puromycin reaction was terminated by addition of 0.5 ml of ammonium bicarbonate 1 M, pH 9 and the ^{14}C-Phe-puromycin formed was extracted with 1 ml ethyl acetate.[14]

Nonenzymic binding of Ac-^{14}C-Phe-tRNA was studied essentially as previously reported[3] by incubation at 37°C for 30 minutes in buffer D, poly U (2 mg/ml), Ac-^{14}C-Phe-tRNA (560 pCi/ml) and ribosomes (3.5 mg/ml).

One ml mixtures were incubated during 45 minutes for the purpose of isolating the complex ribosome-Ac-^{14}C-Phe-tRNA and the isolation procedure after the incubation was as described above for the complex ribosome-^{14}C-Phe-tRNA with the substrate bound to the A-site. Reaction with puromycin of the complex ribosome-Ac-^{14}C-Phe-tRNA was also as indicated above. In the nonenzymic binding of Ac-^{14}C-Phe-tRNA to ribosomes part of the substrate is bound to the P-site as measured by the puromycin reaction but another part is not bound to the P-site. This substrate can be translocated to the P-site by using EF 2 and GTP and separating the translocated complex as described above in the case of the ribosome-^{14}C-Phe-tRNA complex. The extent of the translocation reaction was measured by the increase observed in the subsequent study of the puromycin reaction (Table 1).

The "fragment reaction" assay in which peptide bond formation takes place, catalysed by human tonsil ribosomes reacting CACAC-^{14}C-Leu-Ac with puromycin, was carried out as previously described.[2]

Poly U, dithiothreitol and GTP used were from Sigma, 2-mercaptoethanol from Fluka. Millipore HAWP 2400 (from Millipore Corp.) or Sartorius SM 11306 (Membranfilter GmbH) filters were used. Scintillation fluid prepared by diluting 5 g of butyl-PBD (Ciba) in one liter of toluene was used to measure radioactivity retained by filtration through Millipore filters. To measure ^{14}C-Phe- or Ac-^{14}C-Phe-puromycin extracted by ethyl acetate, a scintillation fluid was used prepared by addition of 250 ml of methoxyethanol to 750 ml of toluene in which 5 g of butyl PBD (Ciba) have been dissolved previously.

Sources of the protein synthesis inhibitors used in this work were as follows: Actinobolin (Parke Davis), amicetin, chartreusin and cycloheximide (Upjohn), adrenochrome and gougerotin (Calbiochem), anisomycin (Pfizer), aurintricarboxylic acid (ATA) (May and Baker), blasticidin S and bottromycin A_2 (Institute of Applied Microbiology, Tokyo, Japan), emetine (Wellcome), fusidic acid

Table 1. The puromycin reaction with Ac-^{14}C-Phe-tRNA bound to ribosomes

Conditions	cpm	pmoles	Translocation pmoles
Ribosomes-Ac-^{14}C-Phe-tRNA + puromycin	717	0.82	—
Ribosomes-Ac-^{14}C-Phe-tRNA + EF2 + GTP + puromycin	1,530	1.75	0.93

Ac-^{14}C-Phe-tRNA was nonenzymically bound to ribosomes and the complex isolated (2.8 pmoles substrate bound per 16 pmoles ribosomes). The complex was treated with EF 2 and GTP and incubated to translocate the substrate bound to the A-site. The complexes prior or after translocation were treated with puromycin and the Ac-^{14}C-Phe-puromycin formed in both cases was extracted with ethyl acetate. Experimental conditions were as indicated under Materials and Methods.

(Leo), puromycin (Serva and Nutritional Biochemicals), sparsomycin (National Cancer Institute, Bethesda, USA) and tenuazonic acid (Merck Sharp and Dohme). Edeine A_1 was a gift from Dr. Z. KURYLO-BOROWSKA (Rockefeller University, New York). Pederine was given to us by Prof. M. PAVAN (Institute of Entomology, University of Pavia, Italia). Diphtheria toxin was a gift from Dr. E. BERMEK (Max-Planck Institute for Experimental Medicine, Göttingen, Germany).

Results

Effect of Protein Synthesis Inhibitors on Substrate Binding to Sites A and P of the Ribosome

The enzymic binding of ^{14}C-Phe-tRNA was carried out to study substrate binding to the A-site of the ribosomes. Our evidence that substrate was bound to the A-site in this assay was that the substrate was not reactive with puromycin after the binding reaction but became reactive (at least 25 % of the bound substrate) after the translocation reaction carried out following the assay described under Materials and Methods. Nonenzymic binding of Ac-^{14}C-Phe-tRNA was also studied and under the experimental conditions used the substrate bound to both the P- and the A-sites as indicated above.

For the purpose of studying the effect of a number of inhibitors in the binding reaction, the required compound was mixed in the incubation mixture with the other components prior to addition of either Ac-^{14}C-Phe-tRNA or GTP which were added to start the reaction in the nonenzymic or enzymic assay respectively. The results obtained are presented in Table 2. Adrenochrome, ATA and edeine A_1 appear to be very effective in blocking binding to both the A- and P-sites of the ribosome. On the other hand although anisomycin, emetine and tenuazonic acid also inhibit to a certain extent binding to the A- and P-sites this is observed at a rather high concentration of the inhibitors.

Table 2. Binding of Ac-^{14}C-Phe-tRNA and ^{14}C-Phe-tRNA to human tonsil ribosomes. Effects of protein synthesis inhibitors

Additions		Ac-^{14}C-Phe-tRNA binding (% control)	^{14}C-Phe-tRNA binding (% control)
Adrenochrome	10^{-4} M	25	23
Anisomycin	10^{-4} M	58	51
	10^{-5} M	99	91
ATA	10^{-4} M	1	15
Edeine A_1	10^{-6} M	26	14
Emetine	10^{-4} M	53	74
Tenuazonic acid	10^{-3} M	67	61
	10^{-4} M	96	92

Assays were carried out using human tonsil ribosomes under the experimental conditions described above. Figures given in this Table are percentage of control reactions in the absence of inhibitor. Binding of Ac-^{14}C-Phe-tRNA in the control reactions was 1.6 pmoles whereas ^{14}C-Phe-tRNA binding was 2.4 pmoles. The following compounds produced no effect or only a marginal difference from the controls in the above reaction assays: Actinobolin (10^{-4} M), amicetin (10^{-4} M), blasticidin S (2×10^{-4} M), bottromycin A_2 (10^{-3} M), chartreusin (2×10^{-4} M), cycloheximide (10^{-4} M), diphtheria toxin (125 μg/ml always assayed in the presence of 10^{-5} M NAD), gougerotin (10^{-4} M), pederine (2×10^{-5} M) and sparsomycin (10^{-4} M).

Table 3. Translocation of Ac-^{14}C-Phe-tRNA and ^{14}C-Phe-tRNA bound to human tonsil ribosomes. Effects of protein synthesis inhibitors

Additions		Ac-^{14}C-Phe-tRNA translocation (% control)	^{14}C-Phe-tRNA translocation (% control)
Cycloheximide	10^{-4} M	—	88
Diphtheria toxin	125 μg/ml	36	11
Fusidic acid	5×10^{-3} M	115	—
	2×10^{-4} M	—	97
Pederine	2×10^{-5} M	32	30

Assays were carried out using human tonsil ribosomes under the experimental conditions indicated under Materials and Methods. Figures given in this Table are percentage of control reactions in the absence of inhibitors. Average of Ac-^{14}C-Phe-tRNA translocated in the controls was 0.8 pmoles (47 % of the total substrate bound) whereas ^{14}C-Phe-tRNA translocated in the control was 0.6 pmoles (21 % of the total substrate bound).

The following compounds produced no effect or only a marginal difference from the controls in the above reation assays: Actinobolin (10^{-4} M), amicetin (10^{-4} M), adrenochrome (10^{-4} M), anisomycin (10^{-4} M), ATA (10^{-4} M), blasticidin S (2×10^{-4} M), bottromycin A_2 (10^{-4} M), chartreusin (2×10^{-4} M), edeine A_1 (10^{-6} M), emetine (10^{-4} M), gougerotin (10^{-4} M), sparsomycin (10^{-4} M) and tenuazonic acid (10^{-4} M).

Effects of Protein Synthesis Inhibitors on the Translocation Reaction

The translocation reaction was studied with either ^{14}C-Phe-tRNA enzymically bound (in this binding reaction all the substrate was bound to the A-site) or Ac-^{14}C-Phe-tRNA

nonenzymically bound (in this binding reaction part of the substrate was bound to the A-site). To test the effect of the different compounds in translocation they were added to the incubation mixture before addition of GTP and the mixture preincubated for 10 minutes at 37°C to facilitate inhibitor binding before starting the translocation reaction by addition of GTP. The effect of protein synthesis inhibitors on the translocation reaction is shown in Table 3. In both systems used there is a clear inhibition in the translocation step by diphtheria toxin and pederine. However no inhibition was found in the in the presence of fusidic acid.

Table 4. Peptide bond formation by human tonsil ribosomes. Effects of protein synthesis inhibitors

		Ac-^{14}C-Phe-Pur formation (% control)	^{14}C-Phe-Pur formation (% control)	Fragment reaction (% control)
Actinobolin	10^{-4} M	94	—	75
	10^{-3} M	—	—	29
Amicetin	10^{-4} M	95	—	—
	10^{-3} M	74	—	—
Anisomycin	10^{-4} M	25	16	5
Blasticidin S	2×10^{-4} M	—	23	—
	10^{-4} M	—	—	12
Cycloheximide	10^{-4} M	—	93	—
Gougerotin	10^{-4} M	97	—	—
	10^{-3} M	—	—	16
Sparsomycin	10^{-4} M	21	19	7
Tenuazonic acid	10^{-4} M	98	50	73
	10^{-3} M	51	—	22

Assays were carired out using human tonsil ribosomes under the experimental conditions described above. Figures given in this Table are percentage of control reactions in the absence of inhibitor. In the control assays 1.75 pmoles Ac-^{14}C-Phe-Pur or 0.68 pmoles ^{14}C-Phe-Pur were formed in the puromycin reaction and 1.39 pmoles Ac-^{14}C-Phe-puromycin were formed in the fragment reaction assay. The following compounds produced no effect or only a marginal difference from the controls in the above reaction assays: Adrenochrome (10^{-4} M), ATA (10^{-4} M), bottromycin A_2 (10^{-4} M), chartreusin (2×10^{-4} M), edeine A_1 (10^{-5} M), emetine (10^{-4} M), diphtheria toxin (125 μg/ml in the presence of 10^{-5} M NAD), fusidic acid (2×10^{-4} M), and pederine (10^{-4} M). (—) shows that the indicated experiment has not been performed.

Effects of Protein Synthesis Inhibitors on Peptide Bond Formation

The effect of protein synthesis inhibitors on peptide bond formation was tested in the following experimental systems: (a) puromycin reaction with ^{14}C-Phe-tRNA prebound enzymically to the A-site and translocated to the P-site prior to addition of puromycin, (b) puromycin reaction with Ac-^{14}C-Phe-tRNA after nonenzymic binding and translocation to position all the substrate in the P-site and (c) ribosome catalysed puromycin reaction with CACCA-^{14}C-Leu-Ac ("fragment reaction"). In all cases the protein synthesis inhibitors were preincubated with the incubation mixtures for 10 minutes at 37°C prior to addition of puromycin to start the reaction. The results obtained in these experiments are presented in Table 4. The results obtained comparing the effect of the different protein synthesis inhibitors in the three systems used are rather in good agreement but the "fragment reaction" appears to be the most sensitive assay to test peptide bond formation inhibitors.

Discussion

The results presented in this contribution show that in human tonsil ribosomes we have a good system to study: substrate binding to the P- and A-sites of ribosomes, translocation and peptide bond formation.

ATA and edeine A_1 were confirmed in this work to be good inhibitors of substrate binding to the ribosome; this finding has repeatedly been observed in other systems[4,15]. In addition we found that adrenochrome is an efficient inhibitor of substrate binding.

Diphtheria toxin and pederine have been clearly shown in this work to be specific inhibitors of translocation. Diphtheria toxin has been assumed in the past years to be an inhibitor of translocation since it acts on EF 2 causing its ADP-ribosylation. However, it has recently been observed that the ADP-ribosyl-EF 2 formed can bind to the ribosome[16] and activate the normal EF 2- and ribosome-dependent GTPase required in translocation.[17] So it is interesting our observation that diphtheria toxin inhibits the translocation step uncoupling it from GTP hydrolysis. We consider very interest-

ing our finding that pederine blocks translocation in the well resolved systems that we have used since this has not been observed previously, although on the basis of some indirect evidence pederine has certainly been postulated as an inhibitor of translocation.[18] On the other hand cycloheximide and fusidic acid do not inhibit translocation in our experimental system. Cycloheximide has previously been reported as an inhibitor of translocation on the basis that it inhibited the increase observed on the puromycin reaction with mammalian polysomes when EF 2 and GTP were added.[19,20] The inhibitory effect of cycloheximide on translocation has also been reported in a resolved reticulocyte system[21] and certainly we cannot understand at the present time the reason for this result differing from those which we present in Table 3 concerning cycloheximide action. Fusidic acid has been reported as an inhibitor of translocation on bacterial[22,23] as well as mammalian systems.[24] This reported inhibition of fusidic acid on translocation was based on either inhibition of GTPase or inhibition of translocation in model systems in certain experimental conditions. However recent experimental evidence in bacterial systems has shown that fusidic acid is not really an inhibitor of translocation[25,26,27,28] and this is also confirmed in this work in a mammalian system.

Anisomycin, sparsomycin and tenuazonic acid have been shown in this work to be good inhibitors of peptide bond formation in all the different experimental systems used. We have already reported inhibition of peptide bond formation by anisomycin and sparsomycin in different eukaryotic systems[1,2,3] but an inhibitory effect of tenuazonic acid on peptide bond formation is described in this contribution for the first time. It is interesting our observation that tenuazonic acid is a good inhibitor of peptide bond formation in mammalian systems but not in yeast.[3]

The antibiotics actinobolin, blasticidin S and gougerotin were found in this work to be very poor inhibitors of peptide bond formation in our model systems for the puromycin reaction but were quite active in blocking peptide bond formation in the fragment reaction which is a more resolved system. We have previously found a similar situation in the case of yeast ribosomes.[3]

The antibiotic amicetin was found in this work to be a very poor inhibitor of peptide bond formation in the two systems used. We have obtained a similar finding in yeast ribosomes but in this case amicetin was quite active in blocking peptide bond formation in the fragment reaction assay performed with 60S subunits[3]. This type of experiment was not carried out in this work.

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