PHOTOINACTIVATION OF PEPTIDYL TRANSFERASE BINDING SITES

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1. Introduction

All efforts to isolate peptidyl transferase from the large ribosomal subunit have thus far failed. Neither isolated ribosomal proteins, nor ribosomal RNAs are able to catalyze peptide bond formation. It is generally accepted that peptidyl transferase consists of a donor and an acceptor substrate binding site on the 50 S ribosomal subunit. Recent studies have suggested that both 23 S rRNA and several ribosomal proteins (i.e., L2, L4, L16) are involved in the structure of these binding sites [1]. On the contrary, there is no conclusive evidence for a catalytic site of ribosomal peptidyl transferase the existence of which is anticipated mainly from analogy with other enzymes (i.e., proteolytic enzymes catalyzing reactions similar to the peptidyl transfer reaction [2,3]).

Inactivation of several essential functions of ribosomes by photooxidation in the presence of sensitizing dyes has been reported [4–6].

In this paper we studied photoinactivation of peptidyl transferase in *Escherichia coli* ribosomes in the presence of eosin or Rose Bengal. To find targets for photoinactivation we investigated catalytic and binding properties of peptidyl transferase in normal and photooxidized ribosomes.

2. Materials and methods

2.1. *Materials*

Puromycin dihydrochloride was obtained from Nutritional Biochemicals, USA. Rose Bengal and eosin were obtained from Lachema, Brno.

L-[4-^{3}H]Phenylalanine (11 Ci/mmol) was a product of the Radiochemical Centre, Amersham. L-[U-^{14}C]-Leucine (126 mCi/mmol) was prepared at the Institute for Research, Production and uses of Radioisotopes, Prague.

2.2. *Preparation of ribosomes*

Ribosomes from *E. coli* B were purified by washing with 1 M NH_4Cl and incubation with puromycin [7].

2.3. *Photooxidation of ribosomes*

Photooxidation of *E. coli* ribosomes was carried out as in [6]. The reaction vessels contained 9 mg ribosomes in 300 μl buffer (30 mM Tris–HCl (pH 7.5), 20 mM $MgCl_2$, 220 mM KCl) with eosin or Rose Bengal, at the concentration indicated, were placed in an ice bath positioned 26 cm from the condensor lens of a 500 W slide projector and irradiated for 20 min. The ribosomes were precipitated with ethanol [8] and resuspended in buffer (10 mM Tris–HCl (pH 7.2), 1 M NH_4Cl, 10 mM $MgCl_2$).

2.4. *Preparation of substrates*

The preparation of [^{3}H]Phe-tRNA, ac[^{3}H]Phe-tRNA and ac[^{14}C]Leu-pentanucleotide was described in [9]. [^{3}H]Phe-pentanucleotide was prepared according to [10]. The 2′(3′)-*O*-(*N*-formylmethionyl)-adenosine-5′-phosphate (pA-fMet) was a gift from Dr A. A. Krayevsky, Institute of Molecular Biology, Moscow and was prepared as in [11].

2.5. *Transfer assay*

Assay of the reaction of ac[^{3}H]Phe-tRNA or ac[^{14}C]Leu-pentanucleotide with puromycin was carried out under the conditions of the fragment reaction in [9]. Acylaminoacyl-puromycin formed was extracted according to [12]. The transfer reaction with pA-fMet and CpApCpCpA-[^{3}H]Phe was assayed as in [11].

2.6. *Binding of acceptor and donor substrates*

The binding of the acceptor substrate, CpApCpCpA-[^{3}H]Phe or [^{3}H]Phe-tRNA, to the ribosomes was determined in the presence of 20% ethanol according to [10].

The binding of the donor substrate, CpApCpCpA-ac[^{14}C]Leu or ac[^{3}H]Phe-tRNA, to the ribosomes was examined as in [13].

3. Results

3.1. *Peptidyl transferase activity of photooxidized ribosomes*

Loss of peptidyl transferase ability to form a new peptide bond in the course of photooxidation with Rose Bengal is shown in fig.1. After 30 min photooxidation, almost complete inactivation is observed in the transfer assay with both donor substrates, acLeu-pentanucleotide and acPhe-tRNA.

The dependence of peptidyl transferase photoinactivation on the concentration of the sensitizing dye is shown in fig.2 and table 1. The results indicate that the effect of Rose Bengal and eosin is similar, but a higher concentration of eosin is necessary to reach the same extent of inactivation (fig.2).

Under the conditions used, neither irradiation of the ribosomes in absence of the dye (fig.1), nor incubation with the dye in the absence of irradiation (fig.2) has any effect on peptidyl transferase activity and the binding of donor and acceptor substrates.

As shown in table 1, the extent of peptidyl transferase inactivation after photooxidation of ribosomes was found to be roughly equal in the transfer reactions with various donor substrates (acPhe-tRNA, acLeu-pentanucleotide or pA-fMet) and acceptor substrates (puromycin or Phe-pentanucleotide).

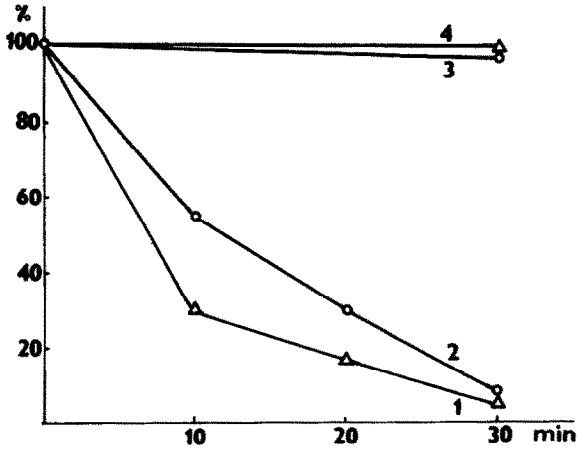

Fig.1. Time course of inactivation of peptidyl transferase activity during photooxidation. The ribosomes were irradiated in the presence of 4×10^{-4} M Rose Bengal for 10, 20 and 30 min and examined in transfer assay with ribosomes (50 pmol) and ac[^{14}C]Leu-pentanucleotide (10 pmol) or ac[^{3}H]Phe-tRNA (2 pmol) as a donor substrate and puromycin (50 nmol) as an acceptor substrate as in section 2. (%) ac[^{14}C]Leu-puromycin formation (100% transfer corresponded to 980 cpm) or ac[^{3}H]Phe-puromycin formation (100% transfer corresponded to 2800 cpm). (1) fragment reaction with the ribosomes, after irradiation in the presence of Rose Bengal, and with ac[^{14}C]Leu-pentanucleotide as a donor substrate; (2) fragment reaction with the ribosomes, after irradiation in the presence of Rose Bengal, and with ac[^{3}H]Phe-tRNA as a donor substrate; (3) fragment reaction with ribosomes, after irradiation without Rose Bengal, and with ac[^{14}C]Leu-pentanucleotide as a donor substrate; (4) fragment reaction with ribosomes, after irradiation without Rose Bengal, and with ac[^{3}H]Phe-tRNA as a donor substrate.

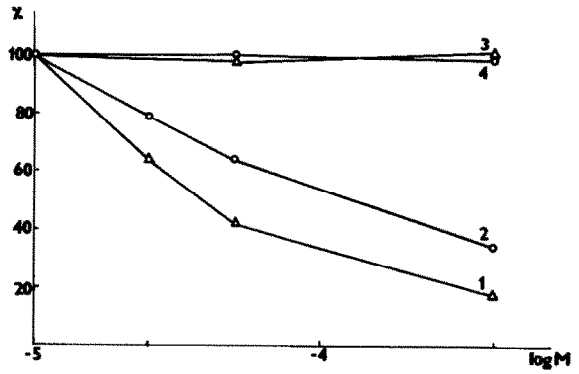

Fig.2. Peptidyl transferase activity of photooxidized ribosomes. The ribosomes were irradiated for 20 min in the presence of different concentrations of Rose Bengal or eosin. Ribosomes (50 pmol) were examined in transfer assay with ac[^{14}C]leucyl-pentanucleotide (10 pmol) as a donor substrate and puromycin (50 nmol) as an acceptor substrate. (%) ac[^{14}C]leucyl-puromycin formation (100% transfer corresponded to 980 cpm); (M) concentration of Rose Bengal or eosin. (1) photooxidation with Rose Bengal; (2) photooxidation with eosin; (3) incubation with Rose Bengal without irradiation; (4) incubation with eosin without irradiation.

Table 1
Transfer reaction of ribosomes, photooxidized in the presence of eosin, with two terminal and one complete donor substrate

Transfer system		Eosin (conc.)	Acylaminoacyl transfer	
Donor substrate	Acceptor substrate		cpm	%
pA-fMet	CpApCpCpA-[^{3}H]Phe	–	2715	100
		5×10^{-5} M	1705	64
		4×10^{-4} M	1038	38
CpApCpCpA-ac[^{14}C]Leu	Puromycin	–	980	100
		5×10^{-5} M	627	64
		4×10^{-4} M	334	34
ac[^{3}H]Phe-tRNA	Puromycin	–	2865	100
		5×10^{-5} M	1718	60
		4×10^{-4} M	1065	37

The ribosomes were irradiated for 20 min in the presence of two concentrations of eosin, precipitated by ethanol and examined in the transfer reaction as in section 2; ribosomes (110 pmol) were examined in the transfer assay with pA-fMet (200 nmol) and CpApCpCpA-[^{3}H]Phe (2 pmol). The transfer assays with CpApCpCpA-ac[^{14}C]Leu and ac[^{3}H]Phe-tRNA were performed under the conditions described in the legend for fig.1

3.2. *Binding of donor substrates to the photoinactivated ribosomes*

In contrast to the peptidyl transferase activity, the donor site is not affected by photooxidation and both the complete donor substrate acPhe-tRNA and the terminal substrate acLeu-pentanucleotide are bound to the same extent even after photooxidation that strongly inactivates peptidyl transferase (table 2).

Table 2
Binding of a terminal and of a complete donor substrate to ribosomes photooxidized in the presence of eosin

Eosin (conc.)	Binding of donor substrate			
	CpApCpCpA-ac[^{14}C]Leu		ac[^{3}H]Phe-tRNA	
	cpm	%	cpm	%
–	1034	100	3126	100
5×10^{-5} M	890	86	3120	100
4×10^{-4} M	783	76	3169	101

The ribosomes were irradiated for 20 min in the presence of two concentrations of eosin and precipitated by ethanol; ribosomes (180 pmol) were tested for binding of donor substrate ac[^{3}H]Phe-tRNA (8 pmol) or CpApCpCpA-ac[^{14}C]-Leu (30 pmol) as in section 2

3.3. *Binding of acceptor substrates to the photoinactivated ribosomes*

As shown in table 3, the binding properties of the acceptor site are profoundly changed by photoxidation. Comparison of the binding of a complete natural acceptor substrate Phe-tRNA and its 3′-terminal fragment CpApCpCpA-Phe indicates that the photooxidized ribosomes lose their ability to interact with

Table 3
Binding of a terminal and of a complete acceptor substrate to ribosomes photooxidized in the presence of eosin

Eosin (conc.)	Binding of acceptor substrate			
	CpApCpCpA-[^{3}H]Phe		[^{3}H]Phe-tRNA	
	cpm	%	cpm	%
–	2614	100	4112	100
5×10^{-5} M	1046	30	3927	96
4×10^{-4} M	573	15	3310	81

The ribosomes were irradiated for 20 min in the presence of two concentrations of eosin and precipitated by ethanol; ribosomes (110 pmol) were examined for binding of the acceptor substrate CpApCpCpA-[^{3}H]Phe (2 pmol) or [^{3}H]Phe-tRNA (2 pmol) as in section 2

the terminal fragment, although the binding of Phe-tRNA is almost unimpaired. The loss of binding ability for Phe-pentanucleotide corresponds to the decrease of the peptidyl transferase activity in photooxidized ribosomes.

4. Discussion

Increasing evidence shows that there are two substrate binding sites on ribosomes, the P-site binding peptidyl-tRNA and the A-site for attachment of aminoacyl-tRNA. Both substrates contain several regions which interact with different parts of the P- and A-ribosomal binding sites. Peptidyl transferase is believed to be a constituent of these binding sites, its donor site (P′) is a part of the ribosomal P-site and its acceptor site (A′) is a part of the ribosomal A-site [14]. P′- and A′-sites interact with the 3′-terminus of peptidyl- or aminoacyl-tRNA, respectively.

The results presented here indicate that photooxidation selectively impairs the acceptor site of peptidyl transferase (A′-site) leaving other parts of the A-site unaffected. This follows from the observation that photooxidized ribosomes do not bind short acceptor substrates of peptidyl transferase such as Phe-pentanucleotide, while the binding of a complete acceptor substrate (Phe-tRNA), containing additional binding sites, is almost unaltered. On the other hand, the ribosomal P-site, including the donor site of peptidyl transferase (P′-site), seems to be completely unaffected by photooxidation of ribosomes as indicated by the same binding ability for both acLeu-pentanucleotide and acPhe-tRNA (table 2).

The decrease in the overall peptidyl transferase activity for formation of a new peptide bond corresponds to the loss of the binding capacity for the acceptor substrate and seems to be caused by this loss. We are therefore inclined to believe that the reaction primarily affected by photooxidation is the binding of the acceptor end CpCpA-aminoacyl of aminoacyl-tRNA to the acceptor site of peptidyl transferase.

This interpretation is in agreement with the results in [15], where inactivation of peptidyl transferase in photooxidized ribosomes was investigated by reconstitution studies, and ribosomal proteins L2, L4 and L16 were identified as targets for photoinactivation. The protein L16 was found to be a constituent of the acceptor site both by reconstitution studies [16] and by affinity labeling [17,18]. Affinity labeling also indicates that the protein L2 is located at the acceptor site [19].

Although efforts have been made to show inactivation of peptidyl transferase without impairing its binding properties and to find an anticipated catalytic site in addition to the defined binding sites, there is no conclusive evidence for a separate catalytic site in the peptidyl transferase. To our knowledge, any observed inhibition of the overall peptidyl transfer reaction is accompanied by a corresponding decrease of substrate binding [13,20–23].

Our results are in contrast to the conclusion in [6], where a loss of peptidyl transferase activity was found to be caused by photooxidation of *E. coli* ribosomes with Rose Bengal and no changes were found in the binding of Phe-tRNA and acPhe-tRNA to the acceptor and donor sites. They interpreted their results as a loss in the catalytic activity of peptidyl transferase without having eliminated interaction of additional ribosomal binding sites with intact molecules of aminoacyl-tRNA or peptidyl-tRNA.

5. Conclusion

The above results indicate that photooxidation of *E. coli* ribosomes in the presence of eosin or Rose Bengal primarily affects the acceptor binding site of peptidyl transferase. The loss of ability for binding the acceptor end of the acceptor substrate causes the inactivation of peptidyl transferase.

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