STRUCTURE–ACTIVITY RELATIONSHIPS OF PUROMYCIN ANALOGUES ON *ESCHERICHIA COLI* POLYSOMES

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1. Introduction

Recent work by Pestka and Hintikka [1] and Pestka [2] on the effect of inhibitors of ribosomal function on the puromycin-dependent release of nascent peptides from bacterial polysomes has revealed substantial differences in the action of these inhibitors on salt-washed ribosomes, polysomes and intact cells. In particular, the peptidyl transferase acceptor site of polysomes has a one hundred-fold greater affinity for puromycin than that of salt-washed ribosomes [2, 3]. In view of these important observations, we have now extended our substrate specificity studies with puromycin analogues [4, 5, 7] to the acceptor site of *E. coli* polysomes to examine the generality of this enhanced affinity.

This work forms part of a wider investigation [4, 5, 7–11] of ribosomal peptidyl transferase, and the action of antibiotic inhibitors on this enzyme, by use of analogues and derivatives of puromycin as low molecular weight structural analogues of the 3′-terminus of aminoacyl-tRNA.

2. Materials and methods

2.1. *Materials*

Lysozyme (Grade I) was obtained from Sigma Chemical Co.; Brij 58 from Atlas Chemical Industries, Delaware; puromycin dihydrochloride from Nutritional Biochemicals Corporation, and [^{3}H] puromycin (specific activity, 2.0 Ci/mmol) from the Radiochemical Centre, Amersham. The 3′-*N*-aminoacyl and 5′-0-nucleotidyl analogues of puromycin were prepared by methods described [10].

2.2. *Methods*

Polysomes were prepared from rapidly growing cultures of *E. coli* MRE600 by a minor modification of the lysozyme-Brij 58 lysis procedure of Pestka and Hintikka [1] and were stored in liquid nitrogen. One minute peptidyl-[^{3}H] puromycin release assays were done essentially as described by Pestka [3], except that in some cases the solution also contained 30% (v/v) methanol and was slightly turbid. The time course of the assay was linear up to at least 1.0 min over the concentration ranges of [^{3}H] puromycin and unlabelled puromycin analogues employed (see table 1).

The pepticyl transferase activity of high-salt-washed *E. coli* MRE 600 ribosomes was assayed by means of the fragment reaction as described by Harris et al. [11].

3. Results and discussion

Since the 15 puromycin analogues used here were not available in radioactive form, the only feasible method of testing them for acceptor substrate specificity in the polysome system was to use these analogues as competitive inhibitors of the formation of polypeptidyl-[^{3}H] puromycin. Analysis of the velocity inhibition data by means of Dixon plots [12] has

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** *Abbreviations*: Pan, puromycin aminonucleoside; Pan-L-amino acid, 3′-*N*-L-aminoacyl-Pan; pPan-L-Phe, 5′-*O*-phosphoryl-Pan-L-Phe; ApPan-Gly, 5′-*O*-adenylyl-Pan-Gly; all other analogues are abbreviated similarly.
Definition: A′-site of peptidyl transferase, that part of the ribosomal acceptor or A-site which is in the active centre of peptidyl transferase [8, 9].

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Table 1
Inhibition constants for puromycin analogues in the release of peptidyl-[^{3}H] puromycin from *E. coli* polysomes

Analogue	$K_i(\mu M)$[1]	K_i/K_m[3]	Concentration range (M)
Pan–Gly	2200	710	$0.5–2.3 \times 10^{-3}$
Pan–L–Leu	200	65	$1.0–4.3 \times 10^{-4}$
Pan–L–Tyr	3.3	1.1	$2.7–11 \times 10^{-5}$
Pan–L–Phe	2.8	0.9	$1.9–8.0 \times 10^{-6}$
Puromycin	3.1[2]	1.0	$0.2–5.0 \times 10^{-6}$
Pan–*O*–benzyl–L–Ser	29	9.4	$2.0–8.2 \times 10^{-5}$
Pan–im–benzyl–L–His	540	170	$0.9–3.9 \times 10^{-4}$
Pan–L–Trp	1400	450	$2.4–9.8 \times 10^{-4}$
Pan–L–Lys	270	87	$0.3–1.4 \times 10^{-4}$
Pan–L–Pro	4000	1300	$0.5–1.9 \times 10^{-4}$
Pan–Gly	2200	710	$0.5–2.3 \times 10^{-3}$
ApPan–Gly	1600	520	$1.4–5.8 \times 10^{-4}$
GpPan–Gly	1200	390	$2.1–8.6 \times 10^{-5}$
UpPan–Gly	830	270	$1.4–5.8 \times 10^{-4}$
CpPan–Gly	61	20	$1.8–7.4 \times 10^{-5}$
CpPan–L–Phe	1.7	0.55	$1,2–5.0 \times 10^{-6}$
pPan–L–Phe	11	3.5	$2.7–11 \times 10^{-5}$
Pan–L–Phe	2.8	0.9	$1.9–8.0 \times 10^{-6}$

[1] Determined with [^{3}H] puromycin as substrate at 0.5 and 5.0 μM. The probable errors computed during least squares fitting of Dixon plots [12] are within the range ±10%. All data were obtained with the same batch of polysomes.
[2] Substrate Michaelis constant, K_m, computed from double reciprocal plot.
[3] The ratio of K_i of each analogue to the K_m of puromycin.

given apparent K_i values for each analogue which are a measure of the relative affinities of these analogues for the A′-site [8, 9] of polysomal peptidyl transferase. Although the equations of the Dixon plot are derived for an enzyme system which obeys Michaelis–Menten kinetics, such equations can, in practice, be used with peptidyl transferase to analyse the two-step puromycin reaction of binding followed by catalysis [1, 2, 13].

The apparent K_i values in the *E. coli* peptidyl-[^{3}H] puromycin release assay for the 15 puromycin analogues are listed in table 1. These data provide, for the first time, a quantitative measure of the contribution made to aminoacyl–tRNA binding by various parts of the peptidyl transferase A′-site. The role of the amino acid side chain is demonstrated by the nine 3′-*N*-aminoacyl analogues. The series of natural amino acids with uncharged side chains confirms the earlier qualitative observations [4–7, 14] that a larger, and hence more hydrophobic [15] side chain gives rise to better binding (smaller K_i), up to a maximum represented by puromycin. This natural antibiotic has presumably evolved to fit optimally into a hydrophobic pocket [7–9] on the ribosome; steric repulsion then may explain the weaker binding of the larger hydrophobic analogues, Pan-*O*-benzyl-L-ser, Pan-im-benzyl-L-His and Pan-L-Trp. The dual hydrophobic–hydrophilic nature of the positively charged lysyl side-chain, and the rigid conformation of proline, may mean that these two analogues do not conform to the simple hydrophobic model. A very similar model has been proposed to explain the donor specificity of peptidyl trnasferase [16].

Of the four Pan-Gly derivatives substituted on the

5′-hydroxyl, only CpPan-Gly showed a marked reduction in K_i relative to that of Pan-Gly. The results are consistent with a binding site on the A′-site of peptidyl transferase which is specific for the penultimate 3′-CMP residue of transfer RNA as suggested previously [5, 7–9]; covalent affinity labelling experiments [11] support the hypothesis that such specificity could arise by conventional base-pairing with the ribosomal RNA. When the glycyl residue of CpPan-Gly was replaced by L-Phe, there was a marked reduction in the K_i value to less than the K_m of puromycin, a result which reflects the dominating effect of the proposed hydrophobic binding site occupied by the benzene ring of phenylalanine [5, 7–9].

The K_m for puromycin in peptidyl–puromycin release from two preparations of *E. coli* polysomes was $3.1–5.2 \times 10^{-6}$ M, essentially the same as the value of 2.4×10^{-6} M obtained by Pestka [2, 3], but about two orders of magnitude below K_m values reported for puromycin with salt-washed ribosomes [2, 3, 17]. Similarly, we have found the K_m values for puromycin and Pan-L-Phe to be 1.1×10^{-4} M and 1.0×10^{-4} M, respectively, with washed ribosomes, 30% methanol and CpApCpCpA-(acetyl-[^{3}H] Leu) as donor substrate in the fragment reaction. Limitations of solubility and availability prevented the determination of fragment reaction K_m values for the other puromycin analogues, but a more qualitative study of their relative reactivities [7] has shown that they conform to a hydrophobic hierarchy similar to that demonstrated here with polysomes. Thus, polysomes appear to have a much higher intrinsic affinity than washed ribosomes for all the puromycin analogues.

The reasons for this difference between polysomes and washed ribosomes are not known, although Pestka [2] has suggested that the presence of native peptidyl-tRNA on the polysome stabilizes a ribosomal conformation with increased affinity for puromycin. Another possibility is that salt washing may remove from polysomes a component which contributes to puromycin binding. The hypothesis [7] that the essential presence of 30% methanol in the fragment reaction substantially weakens the hydrophobic binding of puromycin to the A′-site was not supported by the K_m values for puromycin which were found in the polysome system in the presence and absence of 30% methanol to be 7.5×10^{-6} M and 5.2×10^{-6} M, respectively. Rather, the methanol affected the catalysis of the reaction because it caused a threefold decrease in the V_{max} (results not shown). Further evidence that methanol does not reduce the affinity of peptidyl transferase for puromycin is provided by the similar K_m values determined for puromycin with washed ribosomes in the fragment reaction with CpApApCpCpA-(f-Met) as donor substrate in the presence of 50% methanol (K_m $1.7–1.9 \times 10^{-4}$ M; ref. [2,3]) and in the reaction with acetyl-L-Phe-tRNA as donor substrate in the absence of methanol (3.1×10^{-4} M; ref. [17]).

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