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Competition between tetracycline and tRNA at both P and A sites of the ribosome of *Escherichia coli*

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Fluorescence anisotropy studies performed on 6-demethylchlortetracycline, binding to the ribosome of *E. coli* in competition with tRNA at the P site or at both P and A sites, have provided a quantitative assessment in situ of the interaction of this antibiotic with the A site and have demonstrated that there is also an interaction between tetracycline and the P site.

Fluorescence anisotropy; Tetracycline; Ribosome; A site; P site; Antibiotic

1. INTRODUCTION

Antibiotics of the tetracycline family act upon the ribosome [1]. One of these, 6-demethylchlortetracycline (demeclocycline), is attractive for fluorescence studies because of its relatively high fluorescence quantum yield and its photostability. We have used it already [2] in order to define quantitatively its binding to the ribosome of *Escherichia coli* by the technique of fluorescence anisotropy. The use of an accurate, non-perturbing spectroscopic method appeared to settle highly conflicting data in the earlier literature. We have therefore extended these studies to include the programmed ribosome. The results, described here, support the qualitative assertion [1,3] that tetracyclines act on the ribosomal A site, and they put this onto a quantitative foundation for the first time. But they also show that tetracyclines interact allosterically with the P site, thus raising questions about the mechanism of action of the antibiotic in vivo.

When a demeclocycline molecule binds to a ribosome, its fluorescence anistropy increases, owing to the immobilisation of the fluorophore. By using computer curve fitting to analyse anisotropy values obtained during titrations of ribosomes with demeclocycline, we were able to show clearly [2] that the ribosome possesses one strong binding site for demeclocycline, located on the 30 S subunit, alongside an indeterminate number of weak binding sites on both subunits. Here we describe the effect of previously occupying the tRNA-binding P and A sites.

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2. EXPERIMENTAL

Ribosomes and ribosomal subunits were prepared by standard methods cited elsewhere [2]. 6-Demethylchlortetracycline (Ledermycin® ; demeclocycline) was a kind gift from Cyanamid GmbH (Wolfratshausen, FRG). tRNA was obtained from Boehringer Mannheim and aminoacylated by the method of Traub et al. [4].

Fluorescence titrations were carried out in an SLM 8000 DS spectrofluorimeter. All measurements were made at 25°C. The buffer was 50 mM NH_4Cl, 10 mM Mg acetate, 0.1 mM dithioerythritol, 20 mM Tris-HCl, pH 7.5 (as used in [5]). Details of the procedures for measuring and averaging, of corrections for background and

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of determination of the anisotropy are given elsewhere [2,6].

Mathematical details of the curve fitting, based on application of the law of mass action and Weber's law, are described elsewhere [2]. The equations given in that reference were extended in this work by the addition of terms to cover the greater complexity of the binding pattern of tetracycline to ribosomes in the presence of competition from tRNA.

3. RESULTS AND DISCUSSION

The qualitative effect of occupying the P and A sites successively is shown in fig.1. Demeclocycline in the presence of ribosomes has a high average anisotropy value, since a large percentage of the demeclocycline molecules are bound. When $tRNA_f^{Met}$ is added in the presence of the messenger AUGUUU, so that the P site is occupied, the anisotropy decreases, showing that demeclocycline molecules are displaced. This decrease stops when the number of $tRNA_f^{Met}$ molecules added equals the number of ribosomes (fig.1, left half).

When the P site is occupied and a codon is present in the A site, the latter site may be filled by

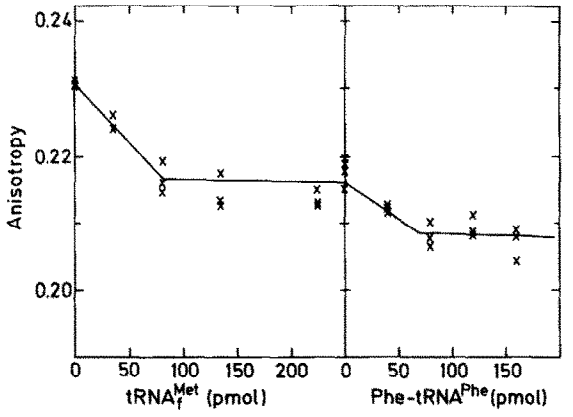

Fig.1. Reduction of fluorescence emission anisotropy by displacement of tetracycline from ribosomes (85 pmol in 250 μl) in the presence of the messenger AUGUUU, elongation factor EF-Tu and GTP. First, the P site is occupied by added $tRNA_f^{Met}$ (left), and then the A site is occupied by Phe-$tRNA^{Phe}$ (right). The results of three titrations are shown; the scatter appears large because of the expanded scale. Controls showed the same effect for the $tRNA_f^{Met}$ titration in the presence of messenger AUG but not in the absence of messenger.

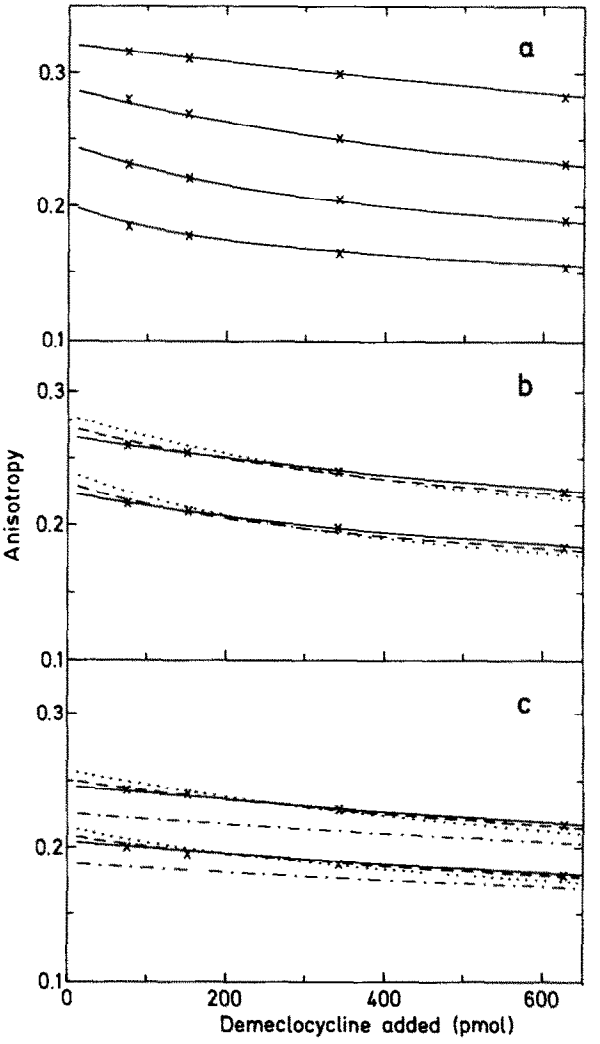

Fig.2. Fluorescence titration of ribosomes with demeclocycline: (a) unoccupied ribosomes, (b) P site occupied, (c) P and A sites occupied. Buffers as in fig.1. Amount of ribosomes in 250 μl at start, lowest to highest curve: (a) 35, 75, 150 and 312 pmol; (b,c) 75, 150 pmol. For practical reasons, not such a wide range of ribosome concentrations could be used in (b) and (c) as in (a). The curves are calculated on the basis of the best-fit values of parameters shown in table 2, computed by individual refinement. For (a), only the established model [2] is considered. For (b), the respective models are: (···) 3, (– – –) 2, (——) 4. For (c), they are: (-·-·-) 5, (···) 7, (– – –) 6, (——) 8. Model 1 gave a very poor fit (cf. residual, table 2) and is omitted. Curves based on global refinement (table 1) are not displayed, but the fit obtained was equally good (cf. residual values in tables 1 and 2).

cognate aminoacyl-tRNA, either with the help of elongation factor EF-Tu and GTP ('enzymically') or, at high magnesium concentration, without them ('non-enzymically'). As the right-hand half of fig.1 shows, the enzymic occupation of the A site is accompanied by a displacement of demeclocycline, again with a stoichiometric ratio of about 1:1. Repetition of the experiment of fig.1 at 20 mM magnesium, with non-enzymic occupation of the A site, gave results identical to those shown here.

In order to interpret the displacement by tRNA in both sites, quantitative titrations were carried out, as shown in fig.2. Consider first fig.2a, obtained with unoccupied ribosomes. As demeclocycline is added (left to right), the percentage of free demeclocycline increases and the average anisotropy decreases. Conversely, the more ribosomes are present (bottom curve to top curve), the higher is the anisotropy throughout.

Fig.2b represents similar experiments repeated in the presence of the AUGUUU messenger and $tRNA_f^{Met}$, i.e. with the P site occupied; fig.2c shows similar experiments where additionally the A site is occupied by Phe-$tRNA^{Phe}$ (enzymic binding).

Curve fitting on the data of fig.2 was carried out in two ways: (i) a single, 'global' refinement of binding parameters to fit the data of the whole of fig.2, and (ii) separate, 'individual' refinement for each of fig.2a,b and c. The former is mathematically more elegant but fails to detect any systematic or local deviation; the latter is cumbrous but gives more detailed insight into the system. Since, if the data are good, either method should give the same result, both were used and will be discussed in turn.

3.1. *Global refinement*

Three models of the mechanism of displacement of demeclocycline by tRNA are considered (table 1). Only the third model, postulating successive weakening of the strong tetracycline-binding site by tRNA in the P and A sites, is acceptable, since (i) the other two give much higher residuals (r values), and (ii) they give K_1 and n_2K_2 values for the unoccupied ribosome which differ substantially from those obtained in our previous work, while

Table 1

Comparison of best fits for different binding models (global refinement)

Model		Result			
		State of ribosomes	$10^{-6}\,K_1$	$10^{-6}\,n_2K_2$	$10^4\,r$
(1)	Removal of weak binding sites by $tRNA_f^{Met}$ and by Phe-$tRNA^{Phe}$	unoccupied		0.81	
		P site occupied	1.25	0.62	180
		A and P sites occupied		0.44	
(2)	1 strong binding site weakened by $tRNA_f^{Met}$, then removed by Phe-$tRNA^{Phe}$	unoccupied	1.11		
		P site occupied	0.43	1.02	105
		A and P sites occupied	0[f]		
(3)	1 strong binding site, weakened successively by $tRNA_f^{Met}$ and Phe-$tRNA^{Phe}$	unoccupied	1.97		
		P site occupied	1.07	0.66	50
		A and P sites occupied	0.45		

[f] Value defined by the model and fixed during refinement.

Parameters displayed were obtained from global curve fitting of the data shown in fig.2. Parameter values underlined are those deemed acceptable by the criterion of best fit. Where one parameter value is given for all three titrations, this means that the refinement was constrained, as required by the model, to give the same value for all three. Results refer to 10 mM magnesium with enzymic occupation of the A site. Units of K_1 and K_2: M^{-1}. The r value is the function of the residuals defined in [2]

the third agrees very well (cf. $K_1 = 2.0 \times 10^6$ M^{-1}, $n_2K_2 = 0.68 \times 10^6$ M^{-1} [2]). Further models were rejected on the same grounds.

3.2. *Individual refinement*

Results are shown in table 2. For unoccupied ribosomes the values of n_1, K_1 and n_2K_2 are the same as those found before (above and [2]).

For ribosomes with the P site occupied, the r value supports unambiguously a weakening of the strong tetracycline binding upon occupation of the P site. Further development of the model (not shown) to allow for the possibility of defective P sites in a heterogeneous population of ribosomes did not show a statistically significant improvement in fit.

For ribosomes with the P and A sites occupied, the r value does not at first allow a clear choice between a reduced number and a reduced strength of the strong tetracycline-binding site, although other models can be excluded. This implies that neither option describes the binding correctly. However, it is known that these preparations typically show an A-site activity of ~65% [5,7,8]. Assuming, therefore, a heterogeneous population of ribosomes in which all the P sites and 65% of the A sites are occupied, we find that the postulated weakening of the tetracycline site gives the best fit by the criterion of the r value (table 2). Repetition with A-site occupation densities of 60 or 70% gave a worse fit.

The titrations represented in fig.2 and the refinement procedures of tables 1 and 2 were repeated for 20 mM magnesium (non-enzymic occupation of the A site). For unoccupied ribosomes, K_1 and n_2K_2 (1.06×10^6 and 0.405×10^6 M^{-1}) agreed well with our earlier values (1.1×10^6 and 0.40×10^6 M^{-1} [2]). For ribosomes with occupied P sites, the best fit was again given by the model involving weakening of the strong tetracycline site with K_1 decreasing to 0.64×10^6 M^{-1} and no detectable heterogeneity in the ribosome population. Addi-

Table 2

Comparison of best fits for different binding models (individual refinement)

State of ribosomes	Model	Result			
		n_1	10^{-6} K_1	10^{-6} n_2K_2	10^4 r
Unoccupied (fig.2a)	one strong and many weak binding sites [2]	1.0	1.98	0.67	50
P site occupied (fig.2b)	(1) elimination of strong tetracycline-binding site[a]	0.0[f]	–	0.67[g]	1200
	(2) reduced number of strong sites[a]	0.67	1.98[g]	0.67[g]	100
	(3) reduced number/strength of weak sites[a]	1.0[f]	1.98[g]	0.512	270
	(4) weakening of strong site[a]	1.0[f]	1.07	0.67[g]	30
P and A sites occupied (fig.2c)	(5) elimination of strong site[b]	0.0[f]	–	0.67[g]	700
	(6) reduced number of strong sites[b]	0.52	1.07[g]	0.67[g]	70
	(7) reduced number/strength of weak sites[b]	1.0[f]	1.07[g]	0.482	200
	(8) weakening of strong site[b]	1.0[f]	0.445	0.67[g]	70
	(9) same, 65% occupation[b,c]	1.0[f]	0.197	0.67[g]	54

Parameters displayed were obtained from curve fitting of the data shown in fig.2a, b or c, respectively. [a] Upon occupation of the P site. [b] Upon occupation of the A site. [c] Repetition with A-site occupation densities of 60% or 70% gave worse fits. [f] Value defined by the model and fixed during refinement. [g] Value obtained from previous experiment and fixed during refinement. For further notes, see table 1

tional occupation of the A site again reduced the strong tetracycline binding, although in this case the less efficient non-enzymic occupation of the A site failed to allow a distinction between weakening and abolition.

We therefore conclude: (i) The ability of demeclocycline, and by inference other tetracyclines, is associated with its single, strong binding site on the 30 S ribosomal subunit. (ii) The antibiotic acts against the P site allosterically, by reducing its affinity for tRNA by some 50%. (This figure follows from the observed change in tetracycline-binding strength upon occupation of the P site.) The functional relevance of this observation should be investigated. (iii) The antibiotic acts against the A site more strongly, reducing its affinity for tRNA to 20% (or possibly somewhat less, if some P sites are defective in table 2). (iv) Since the tRNA-tetracycline interaction occurs on the 30 S ribosomal subunit, which is responsible for codon-anticodon matching [9], it is this function – and region of tRNA – which must be examined further as the target of tetracycline in vivo.

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