HOW DOES *PSEUDOMONAS FLUORESCENS*, THE PRODUCING ORGANISM OF THE ANTIBIOTIC PSEUDOMONIC ACID A, AVOID SUICIDE?

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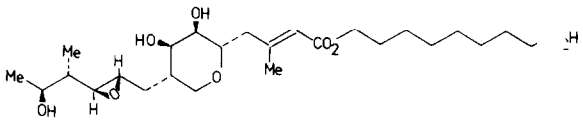
1. Introduction

In [1,2] the target of the novel antibiotic, pseudomonic acid A (fig.1), in the bacterial cell, was shown to be isoleucyl-tRNA synthetase (IRS). The antibiotic is a powerful competitive inhibitor, with respect to isoleucine, of the *Escherichia coli* B enzyme (table 1). It specifically inhibits the formation of the IRS · isoleucyl adenylate complex, as measured by the pyrophosphate exchange reaction (K_i 6 × 10^{-9} M; cf. K_m 6.3 × 10^{-6} M; pH 7.4, 37°C) [3]. The binding ratio of sodium pseudomonate A to IRS is ~1:1. The resulting sodium pseudomonate A · IRS complex is stable and can be isolated by gel filtration on Sephadex G-50. The aminoacylation of yeast $tRNA^{Ile}$, catalysed by rat liver IRS, is also competitively inhibited, with respect to isoleucine, by sodium pseudomonate A [3] but the K_i (20 × 10^{-6} M) is several orders of magnitude higher than that observed for the *E. coli* B enzyme (table 1). It has been suggested [3,4] that the epoxide sidechain terminus of the antibiotic, which has the same carbon skeleton as isoleucine, competes for the amino acid binding site on the enzyme.

The antibiotic has a narrow range of antimicrobial activity which is generally confined to Gram positive bacteria [5]. Most Gram negative organisms, having less penetrable cell walls, are highly resistant to the antibiotic [e.g., minimum inhibitory concentration (MIC) against *Pseudomonas aeroginosa*, >500 μg/ml; cf. MIC against *Staphylococcus aureus*, 0.1 μg/ml].

With the discovery of the target of pseudomonic acid in the bacterial cell, the question arises of how the producing organism of the antibiotic *Pseudomonas fluorescens* (NCIB 10586) [8], is able to survive the fermentation, since the antibiotic is released in the fermentation broth at relatively high levels for a substantial period of the replication phase of the organism [6,7]. There is some evidence from studies with cell wall-deficient mutants of *E. coli* that the significantly greater MIC value for the wild type organism (cf. cell wall mutants) is due, at least in part, to the antibiotics reduced ability to penetrate the cell wall [2]. It is not known, however, to what extent reduced cell wall permeability or structural alteration of the *Ps. fluorescens* enzyme contributes to the relative insensitivity of the organism to the antibiotic.



PSEUDOMONIC ACID A

Fig.1.

Table 1

Source of IRS	Kinetic constants[a] (overall aminoacylation)	
	K_m	K_i
Ps. fluorescens NCIB 10586	9.1 × 10^{-6} M	14.5 × 10^{-3} M
E. coli B	11.1 × 10^{-6} M[b,c]	2.5 × 10^{-9} M[b]
Rat liver	5.4 × 10^{-6} M[b]	2 × 10^{-5} M[b]

[a] pH 7.5, 37°C; [b] from [3]; [c] K_m = 4.6 × 10^{-6} M from [9–13]

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Investigation of the steady state inhibitory kinetics of the aminoacylation of $tRNA^{Ile}$ catalysed by IRS from *Ps. fluorescens* has shown that the enzyme has a markedly reduced affinity for pseudomonic acid A compared to the *E. coli* B and rat liver enzymes.

2. Materials, methods and results

2.1. *Chemicals*

Sodium pseudomonate was kindly donated by Beecham Pharmaceuticals (Chemotherapeutic Research Centre, Brockham Park, Betchworth, Surrey). L-[U-^{14}C]Isoleucine (254 mCi/mmol) was purchased from the Radiochemical Centre (Amersham). Bulk tRNA (from *E. coli* strain W) and amino acids were from Sigma (UK), Fancy Road, Poole, Dorset. Deoxyribonuclease-I and ATP were obtained from Boehringer und Soehne, Mannheim). All other chemicals and solvents were of Analar grade.

2.2. *Radiocounting*

Glass fibre filters were counted in a toluene based scintillant (5 ml) comprising 0.6% butyl PBD and 5% naphthalene, using a Beckman LS-230 liquid scintillation counter (radiocounting efficiency: 85%).

2.3. *Preparation of S100 aminoacyl-tRNA synthetase extract from* Ps. fluorescens

Pseudomonas fluorescens NCIB 10586 was grown in a 50l fermentor as in [8]. Cells were harvested by centrifugation at 10 000 × *g* for 20 min at 4°C, resuspended in 10 mM Tris–HCl buffer (pH 7.8) and recentrifuged. The cells (18 g), alumina (Sigma) (36 g) and Tris–HCl buffer (pH 7.7) (40 ml) were ground for 20 min at 4°C in a mortar and pestle. The paste was centrifuged at 15 000 × *g* for 20 min at 4°C, DNase (final conc. 3 μg/ml) was added to the supernatant and the solution stood for 20 min. The mixture was centrifuged at 30 000 × *g* at 4°C for 1 h and the supernatant recentrifuged at 100 000 × *g* for 3 h at 4°C. The S_{100} preparation was dialysed overnight against Tris–HCl buffer (pH 7.8) and recentrifuged at 35 000 × *g* for 20 min at 4°C. Sucrose (20%, w/w) was added to the supernatant (16 ml) and the aminoacyl-tRNA synthetase preparation stored frozen at −20°C.

2.4. *Aminoacylation assay*

In vitro aminoacylation was measured by the addition of an aliquot (20 μl) of the dialysed S_{100} aminoacyl-tRNA synthetase preparation to the following reagents in 100 μl final vol.: 25 mM Tris–HCl

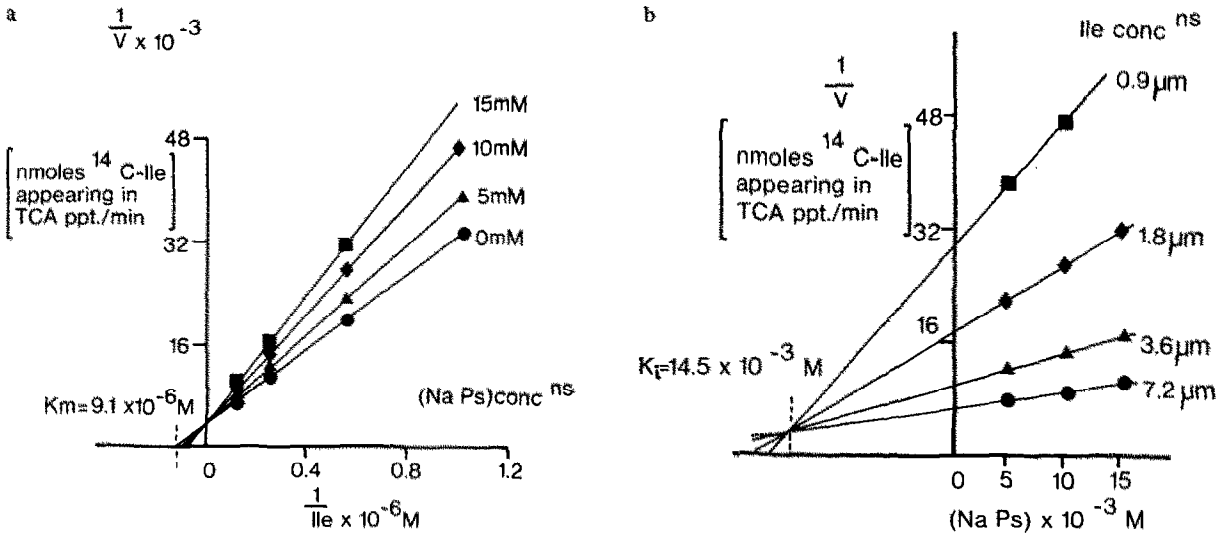

Fig.2. Dialysed S_{100} aminoacyl-tRNA synthetase preparation from *Ps. fluorescens* NCIB 10586 was added to the following reagents in 100 μl final vol.: 25 mM Tris–HCl (pH 7.5); 5 mM ATP, 70 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 250 μg bulk tRNA and L-[U-^{14}C]isoleucine (1–14 μM), and incubated at 37°C for 20 min. After acidification the precipitate was collected and radiocounted. (a) Competitive inhibition of *Pseudomonas fluorescens* (NCIB 10586) IRS: aminoacylation kinetics. Lineweaver-Burk plot: $1/V$ vs $1/[S]$. (b) Dixon plot ($1/V$ vs [I]) of inhibition of $tRNA^{Ile}$ aminoacylation by sodium pseudomonate.

(pH 7.8); 5 mM ATP, 70 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 250 μg bulk tRNA and L-[U-^{14}C]-isoleucine (1–14 μM). After incubation for 20 min at 37°C the reaction was quenched with 7% trichloroacetic acid (1.5 ml) and left at 0–4°C for 30 min. The sample was filtered through a Whatman GF/C glass fibre filter, which was washed successively with 7% trichloroacetic acid (5 × 2 ml) and absolute ethanol (3 × 5 ml). The filters were dried at 100°C for 10 min and radiocounted.

2.5. *Steady state inhibitory kinetics of* Ps. fluorescens *isoleucyl tRNA synthetase*

The rate of isoleucyl-tRNA^{Ile} formation was followed by the above aminoacylation assay at various [^{14}C]isoleucine (1–7 μM) and sodium pseudomonate A (0–15 mM) concentrations. Each experiment was carried out in triplicate and in each case the reaction rate was shown to be linear for ⩾20 min. The results are shown in fig.2.

3. Discussion

As demonstrated for the *E. coli* B and rat liver isoleucyl-tRNA synthetases, the enzyme from the pseudomonic acid A producing organism, *Ps. fluorescens* NCIB 10586, was competitively inhibited by sodium pseudomonate, with respect to isoleucine. It had been established that it is the formation of the IRS · ILE ~ AMP complex catalysed by the *E. coli* B isoleucyl-tRNA synthetase and not the transfer of Ile from the complex to tRNA^{Ile} that is competitively inhibited with respect to isoleucine [3]. For the overall aminoacylation reaction, the Michaelis constants for IRS from the 3 sources (table 1) are of the same order of magnitude. However, the inhibitory constants (K_i) differ significantly. The K_i for the *E. coli* B enzyme is 4000-times lower than the K_m indicating the great affinity of sodium pseudomonate A for the enzyme, whilst the K_m and K_i for the rat liver enzyme are the same order of magnitude. The latter observation clearly indicates that the eucaryotic enzyme has less affinity for the antibiotic than the *E. coli* B enzyme, which is probably the major reason for the low toxicity of the drug in mammals [5]. The K_i for the *Ps. fluorescens* IRS, however, is 1600-times greater than the K_m, and 6 × 10^6-fold greater than the K_i for the *E. coli* B enzyme, clearly indicating that the enzyme from the antibiotic producing organism has a much poorer affinity for the antibiotic. It appears therefore that the *Ps. fluorescens* enzyme is structurally altered compared with IRS from *E. coli* B and the producing organism of the antibiotic avoids suicide, at least in part, by producing a pseudomonic acid-resistant isoleucyl-tRNA synthetase. The extent to which penetration of the antibiotic through the cell wall from the medium, permitting re-entry of pseudomonic acid into the cell during the fermentation, is restricted (and thereby contributing to the ability of the organism to overcome the inhibitory effects of the antibiotic), is not known.

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