Interaction of pseudomonic acid A with *Escherichia coli* B isoleucyl-tRNA synthetase

Julia HUGHES and Graham MELLOWS*

Department of Biochemistry, Imperial College, London SW7 2AZ, U.K.

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Sodium pseudominate was shown to be a powerful competitive inhibitor of *Escherichia coli* B isoleucyl-tRNA synthetase (Ile-tRNA synthetase). The antibiotic competitively inhibits (K_i 6 nM; cf. K_m 6.3 μ M), with respect to isoleucine, the formation of the enzyme • Ile ~ AMP complex as measured by the pyrophosphate-exchange reaction, and has no effect on the transfer of [14 C]isoleucine from the enzyme • [14 C]Ile ~ AMP complex to tRNA^{Ile}. The inhibitory constant for the pyrophosphate-exchange reaction was of the same order as that determined for the inhibition of the overall aminoacylation reaction (K_i 2.5 nM; cf. K_m 11.1 μ M). Sodium [9'- 3 H]pseudominate forms a stable complex with Ile-tRNA synthetase. Gel-filtration and gel-electrophoresis studies showed that the antibiotic is only fully released from the complex by 5 M-urea treatment or boiling in 0.1% sodium dodecyl sulphate. The molar binding ratio of sodium [9'- 3 H]pseudominate to Ile-tRNA synthetase was found to be 0.85:1 by equilibrium dialysis. Aminoacylation of yeast tRNA^{Ile} by rat liver Ile-tRNA synthetase was also competitively inhibited with respect to isoleucine, K_i 20 μ M (cf. K_m 5.4 μ M). The K_m values for the rat liver and *E. coli* B enzymes were of the same order, but the K_i for the rat liver enzyme was 8000 times the K_i for the *E. coli* B enzyme. This presumably explains the low toxicity of the antibiotic in mammals.

We have previously shown (Hughes & Mellows, 1978b) that the primary target of the recently discovered antibiotic pseudomonic acid A (hereafter referred to as pseudomonic acid) in *Escherichia coli* MRE 600 is Ile-tRNA synthetase. In exponentially growing *E. coli* MRE 600 cells treated with the antibiotic at its minimum inhibitory concentration (25 μ g/ml), only 60% of the tRNA^{Ile} was found to be charged with isoleucine, whereas in untreated cells the tRNA^{Ile} remained fully charged. By using a 150 000g supernatant from *E. coli* MRE 600 cells as a source of aminoacyl-tRNA synthetases, it was also shown that 0.2 mM-sodium pseudominate inhibited the overall aminoacylation of tRNA^{Ile} by 90%. With the exception of the aminoacylation of tRNA^{Phe}, which was inhibited by 10% at the same antibiotic concentration, the antibiotic had no effect on the aminoacylation of tRNA^{Gly}, tRNA^{Leu}, tRNA^{Val}, tRNA^{His}, tRNA^{Tyr} and tRNA^{Trp}. We have since shown (G. Mellows,

S. Soughton & K. Taylor, unpublished work) that 0.2 mM-sodium pseudominate also has no effect on the activation of the acidic amino acids aspartic acid and glutamic acid with the same enzyme preparation. However, the same concentration of the antibiotic produced a 70% enhancement in the rate of aminoacylation of tRNA^{Lys}, although the molecular basis for this is unknown. We also demonstrated that sodium pseudominate is a powerful inhibitor of electrophoretically pure *E. coli* B Ile-tRNA synthetase.

In the present paper we describe the steady-state inhibitory kinetics and binding studies of sodium pseudominate with pure *E. coli* B Ile-tRNA synthetase. The inhibitory kinetics of rat liver Ile-tRNA synthetase in relation to the low toxicity of the antibiotic in mammals are also discussed.

Materials and methods

Sodium pseudominate and sodium monate were provided by Beecham Pharmaceuticals Research Division, Chemotherapeutic Research Centre, Brockham Park, Betchworth, Surrey RH3 7AJ, U.K. Electrophoretically pure Ile-tRNA synthetase,

Abbreviation used: Ile-tRNA synthetase, isoleucyl-tRNA synthetase (EC 6.1.1.5).

* Present address: Beecham Pharmaceuticals Research Division, Biosciences Research Centre, Great Burgh, Yew Tree Bottom Road, Epsom, Surrey KT18 5XQ, U.K.

which had been isolated and purified from *E. coli* B, was kindly given by Dr. C. J. Bruton, Department of Biochemistry, Imperial College.

All chemicals and reagents were of AnalaR grade. GF/C glass-fibre filters were from Whatman, Maidstone, Kent, U.K. [$U\text{-}^{14}\text{C}$]Isoleucine (10 Ci/mol), [$U\text{-}^{14}\text{C}$]leucine (150 Ci/mol), [$U\text{-}^{14}\text{C}$]valine (125 Ci/mol), [$U\text{-}^{14}\text{C}$]phenylalanine (522 Ci/mol), NaB^3H_4 (7.2 Ci/mmol) and $\text{Na}_4^{32}\text{P}_2\text{O}_7$ (10 Ci/mol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Sephadex G-50 (fine grade) was from Pharmacia, Uppsala, Sweden. Norit A was from Hopkin and Williams, Chadwell Heath, Essex, U.K. Bulk yeast tRNA, *E. coli* (strain W) tRNA and inorganic pyrophosphatase were obtained from Sigma (U.K.) Ltd., Poole, Dorset, U.K.

Radioactivity counting

This was generally carried out in plastic inserts (5 ml) by using a Beckman LS200B liquid-scintillation counter. Radioactive glass-fibre discs were counted for radioactivity in scintillant A, which contained butyl-PBD [5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] (6 g) and naphthalene (50 g) made up to 1 litre with toluene. Aqueous

samples were counted for radioactivity in scintillant B, which contained methoxyethanol (5 ml) and scintillant A (10 ml).

Glass-fibre discs containing ^{32}P absorbed on Norit A were glued to planchets and counted for radioactivity in a Nuclear-Chicago gas-flow counter.

Synthesis of sodium [$9'\text{-}^3\text{H}$]pseudomonate

The synthetic route to sodium [$9'\text{-}^3\text{H}$]pseudomonate is shown in Scheme 1.

(i) *Preparation of nonan-9-aloic acid.* To oleic acid (1.6 g) was added a solution of KOH (0.8 g) in water (32 ml) with stirring. When the mixture was homogeneous a further portion of KOH (0.8 g) was added and ice (32 g) added, lowering the temperature to $0\text{--}5^\circ\text{C}$. Finely ground KMnO_4 (1.6 g) was added with vigorous stirring and allowed to react for 11 min. The mixture was decolourized with SO_2 , followed by acidification with concentrated HCl . The white precipitate was removed by centrifugation at $5000g$ for 10 min and crystallized from benzene/ethanol (1:2, v/v) to give *erythro*-9,10-dihydroxystearic acid (1.4 g).

A solution of the diol (1.4 g) in ethanol (140 ml) and water (10 ml) was treated at room temperature

Scheme 1.

with a solution of NaIO_4 (3 g) in water (20 ml) for 1 h with stirring. The mixture was filtered, poured into water (500 ml) and the solution adjusted to pH 8 with NaOH . The solution was extracted with diethyl ether (3×100 ml), which was discarded. The aqueous layer was acidified to pH 3 with concentrated HCl and re-extracted with ether (3×100 ml). The combined ether extracts were washed with water, dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure, to give nonan-9-aloic acid (0.5 g): ^1H n.m.r., δ (p.p.m.) 1.39 (6H, broad singlet), 1.65 (4H, multiplet), 2.35 (4H, multiplet), 9.73 (1H, t, J 2 Hz, CHO) and 10.40 (1H, broad singlet, CO_2H).

(ii) *Methyl [9- ^3H]9-hydroxynonanoate*. To a solution of nonan-9-aloic acid (246 mg) in propan-2-ol (10 ml) was added NaB^3H_4 (125 mCi, 7.2 Ci/mmol) and the mixture stirred overnight. Ethanol (20 ml) was then added, followed by NaBH_4 (50 mg), and the mixture stirred for a further 1 h. The mixture was poured into water (150 ml) acidified with 6 M-HCl to pH 3 and extracted with chloroform (3×50 ml). The combined extracts were washed with water (20 ml), dried over anhydrous Na_2SO_4 and the solvent evaporated under reduced pressure, yielding [9- ^3H]9-hydroxynonanoic acid as a white solid. The latter was dissolved in diethyl ether/methanol (1:1, v/v; 2 ml) and an excess of freshly distilled ethereal diazomethane added. The mixture was left overnight and the solvent then evaporated under reduced pressure to give oily methyl [9- ^3H]9-hydroxynonanoate (208 mg).

(iii) *Methyl 9-chloro[9- ^3H]nonanoate*. A solution of methyl [9- ^3H]9-hydroxynonanoate (208 mg), thionyl chloride (0.6 ml) and dry dimethylformamide (one drop) in dry benzene (8 ml) was heated under reflux for 3.5 h. The mixture was evaporated to an oily residue, which was dissolved in chloroform (100 ml) and successively washed with aq. 5% (w/v) NaHCO_3 (20 ml) and water (20 ml). The chloroform solution was dried with anhydrous Na_2SO_4 and the solvent removed under reduced pressure to give methyl 9-chloro[9- ^3H]nonanoate (198 mg) as an oil: ^1H n.m.r., δ (p.p.m.) 1.37 (8H, broad singlet), 1.78 (4H, multiplet), 2.30 (2H, t, J 7 Hz, $-\text{CH}_2\text{OMe}$), 3.51 (2H, t, J 7 Hz, $-\text{CH}_2\text{Cl}$) and 3.67 (3H, s, OMe).

(iv) *Methyl [9'- ^3H]pseudomate*. A mixture of dry sodium monate (204 mg), methyl 9-chloro[9- ^3H]nonanoate (138 mg) and NaI (102 mg) in dry dimethylformamide (2.5 ml) containing hexamethylphosphoramide (one drop) was heated at 100°C with stirring for 3.5 h. The mixture was poured into aq. 5% NaHCO_3 (100 ml) and extracted with ethyl acetate (3×50 ml). The combined extracts were washed with water (50 ml), dried over anhydrous Na_2SO_4 and the solvent was evaporated under reduced pressure to give methyl [9'- ^3H]pseudomate (256 mg) of high purity.

(v) *Sodium [9'- ^3H]pseudomate*. To a solution of methyl [9- ^3H]pseudomate (256 mg) in dimethyl formamide (30 ml) and 0.05 M-sodium phosphate buffer, pH 7.0 (130 ml), was added fresh yeast (6 g) and the suspension stirred at room temperature for 18 h. The mixture was centrifuged at $5000g$ for 20 min and the residual solid washed with ethanol (4 vol.). After re-centrifugation, the combined supernatant and ethanol washings were evaporated to dryness at 40°C under reduced pressure. The residue was dissolved in ethanol (1 ml) and pipetted into 0.1 M-phosphate buffer, pH 8 (50 ml). The solution was extracted with ethyl acetate (2×20 ml), which was discarded. The aqueous phase was acidified to pH 4.0 with 1 M-HCl and re-extracted with ethyl acetate (3×20 ml). The combined extracts were dried with anhydrous Na_2SO_4 , and the solvent was evaporated at reduced pressure to give a pale-yellow oil (205 mg). The oil was purified by preparative layer chromatography on silica gel PF₂₅₄ (Merck) with propan-2-ol/chloroform (3:17, v/v) as eluent. The band with R_f 0.2–0.3 was extracted with methanol to give [9- ^3H]pseudomonic acid (149 mg), which was homogeneous and identical with an authentic specimen by t.l.c. The ^3H -labelled acid was dissolved in methanol (2 ml) and added to water (10 ml). Dilute aqueous NaOH was added to give pH 7.0 and the resulting solution was freeze-dried, yielding sodium [9'- ^3H]pseudomate (151 mg, 53 mCi/mmol) as a foam.

Steady-state kinetic measurements on E. coli B Ile-tRNA synthetase

Overall aminoacylation of Ile-tRNA synthetase. The rate of isoleucyl-tRNA^{Ile} formation was followed by the procedure of Durekovic *et al.* (1973). The reaction mixture (100 μl) contained 100 mM-Tris/HCl, pH 7.5, 2 mM-dithiothreitol, 12 A₂₆₀ units of bulk tRNA, 10 mM-MgCl₂, 10 mM-KCl, 1 mM-ATP, 0.01–0.1 mM-[^{14}C]isoleucine, 0–50 nM-sodium pseudomate and 0.1 μg of Ile-tRNA synthetase. The reaction mixture was pre-equilibrated at 37°C and the reaction timed from the addition of the enzyme. After incubation at 37°C for 3 min, the reaction was quenched by the addition of 5% (v/v) trichloroacetic acid (2 ml) and the mixture kept at 0 – 4°C for 30 min. The precipitate was collected on a Whatman GF/C glass-fibre filter, washed successively with 5% trichloroacetic acid (2×10 ml) and ethanol (2×10 ml), dried and counted for radioactivity in scintillant A.

[^{32}P]Pyrophosphate exchange into ATP. The reaction mixture (1 ml) contained 10 mM-Tris/HCl, pH 7.5, 5 mM-MgCl₂, 10 mM-KF, 2 mM-ATP, 10 mM-dithiothreitol, 2–20 μM -isoleucine, 0–50 nM-sodium pseudomate, 250 μg of Ile-tRNA synthetase and $\text{Na}_4^{32}\text{P}_2\text{O}_7$ (2 μmol ; 10^4 – 10^5 c.p.m./ μmol of pyrophosphate). The reaction mixture was pre-equili-

brated at 37°C and the reaction timed from the addition of the enzyme. After incubation at 37°C for 5 min, the reaction was quenched with 7% (w/v) HClO₄ (0.5 ml) and an aq. 10% (v/v) suspension of Norit A (0.2 ml) added. After 20 min at room temperature, the Norit A was collected on a Whatman GF/C glass-fibre filter. The filter was thoroughly washed with distilled water, glued to a planchet and counted for radioactivity in a gas-flow counter.

Isolation of Ile-tRNA synthetase · [¹⁴C]Ile ~ AMP complex. Ile-tRNA synthetase (500 µg) was added to a reaction mixture containing 2 mM-sodium ATP, 5 mM-MgCl₂, 0.2 unit of inorganic pyrophosphatase (1 µg), 0.1 mM-EDTA, 10 mM-dithiothreitol, 20 mM-[¹⁴C]isoleucine (0.4 µCi) and 20 mM-phosphate buffer, pH 7.5. After 2 min, Blue Dextran was added. The mixture was eluted through a Sephadex G-50 (coarse grade) column (0.5 cm × 28 cm) with 0.1 M-sodium citrate buffer (pH 6.1) containing 0.05 M-KCl and 5 mM-dithiothreitol (the column was pre-equilibrated with the same buffer before use); 160 µl fractions were collected. A portion (10 µl) of each fraction was counted for radioactivity in scintillant B. [¹⁴C]isoleucine was eluted in fractions 40–65, followed by Ile-tRNA synthetase · [¹⁴C]Ile ~ AMP (fractions 25–37). Fractions 25–35 were combined and a portion was used in the following assay.

Effect of sodium pseudomonate on [¹⁴C]isoleucine transfer from Ile-tRNA synthetase · [¹⁴C]Ile ~ AMP complex to tRNA^{Ile}. The Ile-tRNA synthetase · [¹⁴C]Ile ~ AMP complex [20 µl, 2.0 × 10³ d.p.m. (0.12 pmol of complex)] was incubated at 37°C with 20 A₂₆₀ units of bulk tRNA, 10 mM-Tris/HCl, pH 7.2, and 5 mM-KCl with or without sodium pseudomonate in a final volume of 100 µl. After 15 min, the reaction was terminated by the addition of ice-cold 10% trichloroacetic acid (1.5 ml). The mixture was maintained at 0–4°C for at least 15 min before filtration through a Whatman GF/C glass-fibre filter. The filter was successively washed with 5% trichloroacetic acid (20 ml) and ethanol (10 ml), dried and counted for radioactivity in scintillant A.

Separation of Ile-tRNA synthetase · sodium [9'-³H]pseudomonate complex on Sephadex G-50. Sodium [9'-³H]pseudomonate (20 nmol, 53.1 Ci/mol) and Ile-tRNA synthetase (211 pmol) were incubated in 20 mM-Tris/HCl, pH 7.2 (40 µl), containing 10 mM-dithiothreitol for 15 min at 37°C. The mixture was eluted through a Sephadex G-50 (fine grade) column (0.9 cm × 8 cm) with the same buffer. Fractions (90 µl) were collected. A portion (25 µl) of each was counted for radioactivity in scintillant B.

Portions (50 µl) of alternate samples were qualitatively tested for protein by using the procedure of McKnight (1977).

The sodium [9'-³H]pseudomonate · Ile-tRNA syn-

thetase complex was also treated with 0.1% sodium dodecyl sulphate at room temperature and 100°C before gel filtration.

In a separate experiment the Sephadex G-50 column was pre-equilibrated with the same buffer additionally containing unlabelled sodium pseudomonate at the same concentration as that used in the incubation mixture. Gel filtration was then conducted with the same sodium pseudomonate/buffer mixture.

The results are collected in Fig. 5.

Steady-state kinetic measurements on rat liver Ile-tRNA synthetase

Preparation of crude aminoacyl-tRNA synthetase mixture. The procedure of Lanks *et al.* (1971) as modified by Santi & Webster (1976) was used. Two rat livers (21 g) were homogenized (1 min bursts in a Waring Blendor at 0°C) in 1.5 vol. of buffer containing 10 mM-potassium phosphate, pH 7.5, 10 mM-magnesium acetate, 5 mM-mercaptoethanol and 10% (v/v) glycerol. The homogenate was centrifuged at 10000g for 30 min at 4°C. The supernatant was further centrifuged at 200000g at 4°C for 2 h. The resulting post-ribosomal supernatant was dialysed and concentrated overnight against the above buffer also containing 25% glycerol.

Assay of overall aminoacylation of tRNA^{Ile}. In final volume of 100 µl, a portion of the above synthetase preparation (10 µl) was incubated at 37°C for 15 min with 10 mM-Tris-HCl, pH 7.5, 10 mM-magnesium acetate, 10 mM-magnesium acetate, 10 mM-KCl, 2 mM-ATP, 10–200 µM-[¹⁴C]-isoleucine (10 Ci/mol), bulk yeast tRNA, and 0–200 µM-sodium pseudomonate. The reaction mixture was quenched with 5% trichloroacetic acid (1.5 ml) and the precipitate collected and counted for radioactivity on Whatman GF/C filters as described above.

Binding of sodium [9'-³H]pseudomonate to Ile-tRNA synthetase

The stoichiometry and dissociation constant for the binding of sodium [9'-³H]pseudomonate to Ile-tRNA synthetase were determined by equilibrium dialysis. The equipment consisted of a rotating drum containing six cylindrical chambers (volume 800 µl each), each of which was separated by a dialysis membrane. The chambers could be filled or samples removed by a syringe through plugged holes on either side of the membrane. Dialysis-membrane discs were prepared from Visking tubing, boiled in five changes of 5 mM-EDTA followed by boiling in ten changes of water, before use.

Each complete chamber contained, in a final volume of 500 µl: 100 mM-Tris/HCl, pH 7.4, 10 mM-

MgCl₂, 10mM-KCl, 10mM-dithiothreitol and sodium [9'-³H]pseudomonic acid (50–500nM). Ile-tRNA synthetase (414nM) was placed on one side of each chamber. The equipment was continually rotated at 4°C for 18h. Experiments in the absence

of enzyme showed that sodium [9'-³H]pseudomonic acid reached equilibrium across the membrane after 12h. Three samples (100μl) were removed from both sides of each chamber and counted for radioactivity in scintillant B.

Results

Previous work has shown that sodium pseudomonic acid causes arrest of protein synthesis in prokaryotic organisms both *in vivo* and *in vitro* by specifically interfering with the function of isoleucyl-tRNA synthetase (Hughes & Mellows, 1978a,b). The highly purified *E. coli* B enzyme was

Fig. 1. Overall aminoacylation kinetics of isoleucyl-tRNA^{Ile} formation by *E. coli* B Ile-tRNA synthetase. The rate of isoleucyl-tRNA^{Ile} formation was determined at 37°C and pH 7.5, over a 3 min period at variable [¹⁴C]isoleucine (0.01–0.1μM) and sodium pseudomonic (0–50nM) concentrations as described in the Materials and methods section. (a) Lineweaver-Burk plot (K_m 11.1μM); (b) Dixon plot (K_i 2.5nM).

Fig. 2. Kinetics of [³²P]pyrophosphate exchange into ATP.

The rate of [³²P]pyrophosphate exchange into ATP was determined at 37°C and pH 7.4 over a 5 min period at variable isoleucine (2–20μM) and sodium pseudomonic (0–50nM) concentrations as described in the Materials and methods section. (a) Lineweaver-Burk plot (K_m 6.3μM); (b) Dixon plot (K_i 6.0nM).

Table 1. Kinetic constants (pH 7.4, 37°C) of isoleucyl-tRNA synthetase

Source of Ile-tRNA synthetase	ATP/[³² P]-pyrophosphate exchange		Overall aminoacylation	
	K_m (μ M)	K_i (Ile) (μ M)	K_m (μ M)	K_i (Ile) (μ M)
<i>E. coli</i> B	6.3*	6.0×10^{-3}	11.1†	2.5×10^{-3}
Rat liver	—	—	5.4	20
<i>Ps. fluorescens</i> N.C.I.B. 10586	—	—	9.1	14.5×10^3

* K_m (literature) 4.0μ M (Rainey *et al.*, 1977).

† K_m (literature) $4-6 \mu$ M (Cole & Schimmel, 1970; Fersht & Kaethner, 1976; Hélène *et al.*, 1971; Loftfield & Eigner, 1965; Rainey *et al.*, 1977).

Table 2. Effect of sodium pseudomonalate on [¹⁴C]-isoleucine transfer from Ile-tRNA synthetase·[¹⁴C]Ile ~ AMP complex to tRNA^{Ile}

Ile-tRNA synthetase·[¹⁴C]Ile ~ AMP complex (0.12 pmol, 2000 c.p.m.), 20 A_{260} units of bulk tRNA, 10 mM-dithiothreitol, 20 mM-Tris/HCl, pH 7.2, and 5 mM-KCl, in a final volume of 100 μ l, were incubated at 37°C for 15 min in the presence or absence of sodium pseudomonalate. The trichloroacetic acid-precipitable material was collected on a glass-fibre filter and counted for radioactivity.

Sodium pseudomonalate concn. (mM)	[¹⁴ C]Isoleucyl-tRNA ^{Ile} collected on filter (c.p.m.)
0	548
0.2	546
2.0	636

shown to be markedly inhibited by the antibiotic, although details of the type of inhibition were not discussed.

Steady-state inhibitory kinetics of *E. coli* B Ile-tRNA synthetase

The data are summarized in Figs. 1 and 2. The important points are:

(a) the overall aminoacylation of tRNA^{Ile} and the pyrophosphate-exchange reaction were competitively inhibited by sodium pseudomonalate in the concentration range 0–50 nM, with respect to isoleucine;

(b) the Michaelis constants, K_m (11.1 μ M and 6.3 μ M respectively), for the two reactions with isoleucine in the concentration range $0.01 < [\text{Ile}] < 0.1$ mM are close to the previously reported values (Table 1);

(c) the inhibitory constants, K_i , for both reactions (2.5 nM and 6 nM respectively) are several orders of magnitude lower than the corresponding K_m values.

Transfer of [¹⁴C]isoleucine from Ile-tRNA synthetase·[¹⁴C]Ile ~ AMP complex to tRNA^{Ile}

The *E. coli* B Ile-tRNA synthetase·[¹⁴C]-

Fig. 3. Stoichiometry of binding of sodium [9'-³H]-pseudomonalate to Ile-tRNA synthetase measured by equilibrium dialysis

Ile-tRNA synthetase (414 nM) and sodium [9'-³H]pseudomonalate (50–5000 nM) were separately placed on each side of an equilibrium-dialysis chamber containing 10 mM-MgCl₂/10 mM-KCl/10 mM-dithiothreitol/100 mM-Tris/HCl, pH 7.4, in a total volume of 500 μ l (as described in the Materials and methods section). The chambers were continually rotated at 4°C for 18 h. Three samples were removed from both sides of each chamber and counted for radioactivity. r = mol of [³H]pseudomonalate bound/mol of Ile-tRNA synthetase. Binding ratio [³H]pseudomonalate/Ile-tRNA synthetase = 0.85:1.

Ile ~ AMP complex was isolated by gel filtration on Sephadex G-50 after incubation of the enzyme with an excess of [¹⁴C]isoleucine and ATP in the presence of inorganic pyrophosphatase. Sodium pseudomonalate had no effect on the transfer of [¹⁴C]isoleucine from the complex to tRNA^{Ile} (Table 2).

Equilibrium dialysis

A Scatchard plot (Fig. 3) revealed a molar binding ratio for sodium pseudomonic acid to Ile-tRNA synthetase of 0.85:1, which is probably more reliable than that obtained by gel filtration and clearly suggests a 1:1 binding ratio. The dissociation constant, $K_{\text{diss.}}$, was found to be 75 nM and the association constant, $K_{\text{ass.}}$, 1.3×10^7 .

Fig. 4. Overall aminoacylation kinetics of isoleucyl-tRNA^{Ile} formation by rat liver Ile-tRNA synthetase. The rate of isoleucyl-tRNA^{Ile} formation was determined at 37°C and pH 7.5, over a 15 min period at variable [¹⁴C]isoleucine (10–200 μM) and sodium pseudomonic acid (0–200 μM) concentrations by using a crude 200000 g rat liver supernatant as a source of Ile-tRNA synthetase as described in the Materials and methods section. (a) Lineweaver–Burk plot (K_m 5.4 μM); (b) Dixon plot (K_i 20 μM).

Steady-state inhibitory kinetics of rat liver Ile-tRNA synthetase

The results are depicted in Fig. 4. As was demonstrated for the *E. coli* B enzyme, sodium pseudomonic acid, in the concentration range 0–200 μM, is a competitive inhibitor of the rat liver Ile-tRNA synthetase, with respect to isoleucine. The inhibitory constant (K_i , 20 μM), is four times the Michaelis constant (K_m , 5.4 μM), and is 8000 times the K_i for the *E. coli* B enzyme.

Fig. 5. Gel filtration of sodium [^{9'}-³H]pseudomonic acid-Ile-tRNA synthetase complex.

Ile-tRNA synthetase (211 pmol) was incubated at 37°C with sodium [^{9'}-³H]pseudomonic acid (20 nmol, 1.18×10^5 d.p.m.) in 20 mM-Tris/HCl buffer, pH 7.2, containing 10 mM-dithiothreitol for 15 min. The mixture was eluted through a Sephadex G-50 (fine grade) column (0.8 cm × 8 cm) with the same buffer, after treatment (a), (b) or (c); (a) reaction mixture directly applied to column ([³H]pseudomonic acid-enzyme complex contained 17816 d.p.m., i.e. 154 pmol of bound [³H]pseudomonic acid); (b) reaction mixture treated with 0.1% sodium dodecyl sulphate for 10 min at 24°C before gel filtration ([³H]pseudomonic acid-enzyme complex contained 11888 d.p.m., i.e. 107 pmol of bound [³H]pseudomonic acid); (c) reaction mixture boiled with 0.1% sodium dodecyl sulphate for 10 min before gel filtration.

Stoichiometry of sodium pseudominate binding to E. coli B Ile-tRNA synthetase

Gel filtration. When Ile-tRNA synthetase and an excess of sodium [9'-³H]pseudominate were incubated at pH 7.2 in the presence of dithiothreitol, followed by gel filtration on Sephadex G-50 (fine grade) pre-equilibrated and eluted with the same buffer containing the same concentration of non-radioactive sodium pseudominate, the molar binding ratio of sodium [9'-³H]pseudominate to Ile-tRNA synthetase was found to be 0.73:1 (Fig. 5a). The same result was obtained when non-radioactive sodium pseudominate was excluded from the eluting buffer, confirming that the sodium [9'-³H]pseudominate-Ile-tRNA synthetase complex is extremely stable and that no equilibrium of the labelled antibiotic in the complex with unlabelled antibiotic in the eluent buffer took place on gel filtration in the former experiment. Inclusion of 50 μ M-ATP in the reaction mixture did not change the binding ratio. Addition of 0.1% sodium dodecyl sulphate at 24°C to the reaction mixture before gel filtration resulted in partial breakdown of the antibiotic-Ile-tRNA synthetase complex, lowering the molar binding ratio to 0.5:1 (Fig. 5b). Boiling in 0.1% sodium dodecyl sulphate resulted in the complete fragmentation of the antibiotic-Ile-tRNA synthetase complex (Fig. 5c). When sodium [9'-³H]pseudominate was incubated with bovine serum albumin in lieu of Ile-tRNA synthetase at the same molar concentration, no binding was observed on gel filtration. This implied that the binding of sodium pseudominate to Ile-tRNA synthetase is specific and not random.

Table 3. *Effect of sodium pseudominate on aminoacylation of tRNA species in vitro with a rat liver aminoacyl-tRNA synthetase preparation*

By using a dialysed 200 000 g supernatant from rat liver as a source of aminoacyl-tRNA synthetases, the aminoacylation of different tRNA species with the corresponding U-¹⁴C-labelled amino acid was determined in the presence and absence of 0.2 mM-sodium pseudominate (100 μ g/ml) as described in the Materials and methods section. The degree of inhibition of aminoacylation was calculated from the incorporation of ¹⁴C into trichloroacetic acid-precipitable material in the presence and absence of sodium pseudominate.

Amino acid	Inhibition of aminoacylation by sodium pseudominate (%)
Isoleucine	60
Phenylalanine	10
Valine	0
Leucine	0

Effect of sodium pseudominate on other rat liver aminoacyl-tRNA synthetases

In addition to producing a marked inhibitory effect on *E. coli* Ile-tRNA synthetase, sodium pseudominate also weakly inhibits the function of phenylalanyl-tRNA synthetase from the same organism (Hughes & Mellows, 1978b).

Interestingly, 0.2 mM-sodium pseudominate also weakly inhibits rat liver phenylalanyl-tRNA synthetase (Table 3), whereas the antibiotic was without effect on leucine and valine activation.

Discussion

We have previously shown that sodium pseudominate is a powerful inhibitor of Ile-tRNA synthetase from *E. coli* MRE 600. To date, Ile-tRNA synthetase has been purified to homogeneity from three *E. coli* strains, K12, MRE 600 and B. The enzyme from all three sources is a single polypeptide chain (Fayat *et al.*, 1974; Durekovic *et al.*, 1973; Arndt & Berg, 1970). The reported molecular weight of the *E. coli* B and K12 enzymes is 110 000 (Baldwin & Berg, 1966; Fayat *et al.*, 1974), whereas the *E. coli* 600 enzyme has a reported molecular weight of 102 000 (Durekovic *et al.*, 1973). The three distinct enzymes appear to be structurally different, as manifested by minor differences in amino acid composition (Table 4) and the demonstration of different N-terminal sequences for the *E. coli* B and MRE 600 enzymes (Durekovic *et al.*, 1973). The reaction catalysed by *E. coli* K12 Ile-tRNA synthetase has been conclusively shown to occur in two discrete stages through the intermediacy of an enzyme-isoleucyladenylate complex (Fersht & Kaethner, 1976):

Identification of the rate-determining step in the synthesis of Ile-tRNA^{Ile} has been the subject of several studies, but the situation is far from clear. Current evidence (Fersht & Kaethner, 1976) suggests that the rate-determining step for the *E. coli* K12 enzyme is the transfer of the isoleucyl moiety from the enzyme-Ile~AMP complex to tRNA^{Ile}. Yarus & Berg (1969) and Eldred & Schimmel (1972) have presented evidence supporting the dissociation of the aminoacylated tRNA from the *E. coli* B enzyme as the rate-determining step, but the opposite conclusion was reached in a more recent study (Lövgren *et al.*, 1976). All three enzymes have a requirement for an active thiol group, and the sequence surrounding the cysteine residue in the *E. coli* MRE 600 enzyme has been reported (Kula,

Table 4. *Amino acid composition of E. coli isoleucyl-tRNA synthetases*
Abbreviation: ND, not determined.

Amino acid	<i>E. coli</i> Reference	Residues (mol/mol of enzyme)		
		B (Durekovic <i>et al.</i> , 1973)	MRE 600 (Baldwin & Berg, 1966)	K12 (Fersht & Kaethner, 1976)
Tryptophan	...	19	17	ND
Lysine	...	61	60	59
Histidine	...	26	23	31
Arginine	...	45	44	39
Half-cystine	...	14	14	14
Aspartic acid	...	88	90	94
Threonine	...	46	44	49
Serine	...	44	42	52
Glutamic acid	...	91	90	96
Proline	...	47	43	44
Glycine	...	72	75	81
Alanine	...	93	91	93
Valine	...	59	70	79
Methionine	...	23	22	23
Isoleucine	...	48	44	47
Leucine	...	83	73	81
Tyrosine	...	32	30	39
Phenylalanine	...	31	29	36

1974). It has also been demonstrated that the *E. coli* B enzyme has one binding site for each of the three substrates (Norris & Berg, 1964; Yarus & Berg, 1967, 1969). In the present study we have shown that sodium pseudomonic acid is a competitive inhibitor, with respect to isoleucine, of the overall aminoacylation of tRNA^{Ile} catalysed by the *E. coli* B enzyme (K_i , 2.5 nM; K_m , 11 μ M) and that it is specifically the formation of the enzyme · Ile ~ AMP complex, as measured by the pyrophosphate-exchange reaction, that is competitively inhibited (K_i , 6 nM; K_m , 6.3 μ M). The transfer of [¹⁴C]isoleucine from purified Ile-tRNA synthetase · [¹⁴C]Ile ~ AMP complex to tRNA^{Ile} was not inhibited by sodium pseudomonic acid. The significantly lower (1000-fold) K_i than K_m value for the formation of the Ile-tRNA synthetase · Ile ~ AMP complex emphasizes the potency of the antibiotic for the enzyme.

We and others (Hughes & Mellows, 1978a; Sutherland *et al.*, 1976) have previously noted that sodium pseudomonic acid is relatively specific for prokaryotic organisms and has a relatively low potency towards eukaryotic organisms. This prompted us to examine the effect of the antibiotic on the mammalian enzyme. The steady-state inhibitory kinetics of the rat liver Ile-tRNA synthetase was also found to be competitive with respect to isoleucine. The inhibitory constant (K_i , 20 μ M), however, was of the same order of magnitude as the Michaelis constant (K_m , 5.4 μ M). The significantly higher K_i value for the mammalian enzyme (cf. *E. coli* enzyme) is clearly a major factor in explaining

the low toxicity of the drug in mammals (i.e. sodium pseudomonic acid has a lower affinity for the mammalian Ile-tRNA synthetase).

In more recent work (Hughes *et al.*, 1980) it has been shown that Ile-tRNA synthetase isolated from the antibiotic-producing organism *Pseudomonas fluorescens* N.C.I.B. 10586 has a K_m value for isoleucine (Table 1) which is comparable in magnitude with the K_m values for the rat liver and *E. coli* B enzymes. The K_i value, however, is 725 times that for the rat liver enzyme, indicating that the *Pseudomonas* Ile-tRNA synthetase is highly resistant to the antibiotic. This is probably a major reason why this organism is able to survive in the fermentation medium, which contains significant amounts of the antibiotic during the later stages of the fermentation process.

Equilibrium-dialysis and gel-filtration studies clearly show that sodium [9-³H]pseudomonic acid binds to *E. coli* B Ile-tRNA synthetase in a molar ratio of approx. 1:1, with an association constant ($K_{ass.}$) of 1.3×10^7 and a dissociation constant ($K_{diss.}$) of 75 nM.

We have previously suggested (Hughes & Mellows, 1978b,c) that the terminus of the side chain containing the epoxide group in the antibiotic, which has the same carbon skeleton as L-isoleucine, competes for the single isoleucine-binding site (Norris & Berg, 1964) on the enzyme (Fig. 6). Although the observed molar binding ratio for Ile-tRNA synthetase and sodium pseudomonic acid is in accord with this notion, one cannot exclude the

Fig. 6. Interaction of pseudomonic acid (a) and isoleucine (b) with the isoleucine-binding site on isoleucyl-tRNA synthetase
(Redrawn from Hughes & Mellows, 1978b.)

alternative possibility that the antibiotic binds at a site close to the isoleucine-binding site, thereby preventing isoleucine entry to the site. It seems unlikely to us that this would be the case, since it is difficult to see how an excess of isoleucine would counteract the presumed highly specific binding of sodium pseudomunate, unless isoleucine could also bind at the second site and its binding constant for this site is comparable with that of the antibiotic. To date, there is no evidence for a second isoleucine-binding site which is capable of binding isoleucine to this extent.

The results of two previous investigations in which the binding of structurally related compounds to isoleucine, ATP and pyrophosphate to Ile-tRNA synthetase was studied (Holler *et al.*, 1973; Flossdorf *et al.*, 1976) have provided strong evidence for the presence of two hydrophobic sites, respectively accommodating the methyl and ethyl groups of isoleucine in the isoleucine-binding domain of the enzyme. Holler *et al.* (1973) further proposed that entrance to the isoleucine-binding domain is an ion-pair 'gate', and that prebinding of ATP at a site in close proximity to the isoleucine-binding domain assists the opening of the ion-pair interaction of the α -ammonium and carboxylate groups of isoleucine with the respective negative and positive components of the ion-pair and would serve to stabilize the Ile-tRNA synthetase $\cdot$ ATP $\cdot$ Ile complex. It was also suggested (Holler *et al.*, 1973) that opening of the ion-pair gate could initiate the conformation change that was observed to follow the binding of isoleucine to the enzyme (Yarus & Berg, 1969; Holler *et al.*, 1971; Holler & Calvin, 1972).

In the light of these observations it was decided to see whether ATP prebinding had any effect on the extent of sodium pseudomunate binding. Pretreat-

ment of Ile-tRNA synthetase with 10 mM-ATP and 10 mM-MgCl₂ had no effect on the degree of binding of the antibiotic. Given that sodium pseudomunate competes for the isoleucine-binding site of the enzyme, as the above evidence clearly suggests, and that the epoxide side-chain terminus of the molecule occupies the two hydrophobic domains, then the 'ion-pair gate' hypothesis of Holler *et al.* (1973) must be questioned. It is not, however, beyond the bounds of possibility that, if the proposed ion-pair interaction in native Ile-tRNA synthetase is weak, that the many functionalities in the rest of the antibiotic molecule may be suitably juxtaposed to associate with suitable groups on the enzyme backbone through non-covalent interactions of sufficient total strength to force the epoxide side-chain terminus into the isoleucine-binding domain.

The affinity of the *E. coli* B enzyme for sodium pseudomunate was further emphasized by the results of Sephadex G-50 gel-filtration studies. The sodium pseudomunate $\cdot$ Ile-tRNA synthetase complex is stable to gel filtration and can be readily isolated. Treatment of the complex with 0.1% sodium dodecyl sulphate at 24°C results in only partial release of the antibiotic from the complex. Severe denaturing conditions, such as boiling the complex in 0.1% sodium dodecyl sulphate or treatment with 5 M-urea, are necessary to release the antibiotic fully from the complex, presumably through complete unfolding of the protein chain. It was rather surprising that the results of the above experiments and the kinetic studies have shown that the epoxide moiety of the antibiotic is not involved in covalent-bond formation with the enzyme, particularly since it is known that there is an active and essential cysteine thiol residue at or close to the active site(s) (Iaccarino & Berg, 1969).

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