FLUORINATED TRYPTOPHANS AS SUBSTRATES AND INHIBITORS OF THE ATP–[^{32}P]PP_i EXCHANGE REACTION CATALYSED BY TRYPTOPHANYL tRNA SYNTHETASE

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1. Introduction

An approach to the study of specific interactions between the aminoacyl tRNA synthetases and amino acids is the investigation of the ability of different amino acid analogues to inhibit or the stimulate the ATP–[^{32}P]PP_i exchange reaction or aminoacyl tRNA synthesis. Substitution of the hydrogen atoms in the substrates by fluorine makes it possible to study the role of the electron factors in the 'recognition' and catalytic conversion of the substrates since the size of the fluorine atom is close to that of hydrogen whereas its electronegativity value is quite distinct. Correlation between the affinity for the enzyme and electron characteristics of the substrate has been found for a number of fluorinated phenylalanine analogues [1,2].

Tryptophanyl tRNA synthetase (tryptophan: tRNA ligase, EC 6.1.1.2) has not previously been studied in detail in this respect. However, it has been shown that D,L-5-fluoroTrp† and D,L-6-fluoroTrp are active in aminoacyl hydroxamate formation reaction; also D,L-6-fluoroTrp stimulates the ATP–[^{32}P]PP_i exchange reaction [3]. Moreover, it is known that 4,5,6,7-tetrafluoroTrp inhibits both the tryptophanylhydroxamate and aminoacyl tRNA formation [4].

The purpose of this study was the detailed investigation of the interactions of fluorotryptophans with beef pancreas TRSase. It has been found that all the monofluorotryptophans act as substrates in the ATP–[^{32}P]PP_i exchange reaction whereas the polyfluorotryptophans under study competetively inhibit this reaction. Correlations have been obtained between the kinetic parameters of the reaction and the position of the fluorine atom substituent in the tryptophan molecule.

2. Materials and methods

2.1. *Materials*

[^{32}P]Disodium pyrophosphate was a product of 'Isotop' (USSR). The following chemicals were used: ATP disodium salt ('Reanal'); Tris ('Koch-Light'); D-Trp ('Calbiochem'); activated charcoal Norit A ('Serva'); pig kidney D-amino acid oxidase ('Schuchardt'); ultrafilters 'RUFS' ('Chemapol'); Sephadex G-50 ('Pharmacia').

The preparation of TRSase has been described [5]. The enzyme form E_2 (mol. wt. 120 000) was used, it was homogeneous in the polyacrylamide gel electrophoresis. The preparation was passed through a Sephadex G-50 column equilibrated with 0.05 M

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† *Abbreviations used:* TRSase, tryptophanyl transfer ribonucleic acid synthetase; Trp, tryptophan; 4-fluoroTrp, 4-fluorotryptophan (other fluorinated analogues of tryptophan are designated in similar way; numbers show positions of the fluorine atoms in the indole ring); BSA, bovine serum albumin.

Tris–HCl buffer pH 7.5 and stored in 30% ethylene glycol at −15°C.

2.2. Synthesis of fluorinated tryptophans

5-FluoroTrp and 6-fluoroTrp were synthesized by the procedures [6] and [7], respectively. 4- and 7-fluoroTrp, similarly to 6-fluoroTrp [7], were obtained from the corresponding fluoroindols by conversion into 4- and 7-fluorogramins, condensation with acetamidomalonic ester and subsequent hydrolysis and partial decarboxylation of the diethyl (fluoroscatyl) acetamidomalonates formed. Hydrochloric solutions of the 4- and 7-fluoroTrp were passed through a Dowex 50W × 8 column and recrystallized from 30% aqueous alcohol. Another method has been described for synthesis of 4-fluoroTrp [8]. *7-Fluorogramin*, yield 70%, mp 134–138°C (ethanol). *Anal.* Calcd. for $C_{11}H_{13}FN_2$: C, 68.75; H, 6.77; F, 9.9; N, 14.60. Found: C, 68.13; H, 6.83; F, 9.5; N, 14.38. *Diethyl (7-fluoroscatyl)-acetamido malonate*, yield 75%, mp 126.5–128°C (ethanol). *Anal.* Calcd. for $C_{18}H_{21}FN_2O_5$: F, 5.2; N, 7.69. Found: F, 4.9; N, 7.30. *7-FluoroTrp*, mp 254.5–256°C. *Anal.* Calcd. for $C_{11}H_{11}FN_2O_2$: C, 59.46; H, 4.95; N, 12.60. Found: C, 59.41; H, 5.11; N, 12.37. *4-FluoroTrp*, mp 274–275°C (lit. mp 241°C [8]). *Anal.* Calcd. for $C_{11}H_{11}FN_2O_2$: C, 59.46; H, 4.95; F, 8.6; N, 12.60. Found: C, 58.51; H, 5.02; F, 9.3; N, 12.28.

4,5,6,7-TetrafluoroTrp was obtained by condensation of the 4,5,6,7-tetrafluoro-3-(*N*-piperidine methyl)-indole (or its dimethyl sulphate quaternary salt) with acetoamide malonic ester and subsequent hydrolysis in 20% hydrochloric acid [9].

5,7-DifluoroTrp was prepared following the method of Fischer: cyclization of γ-acetamido-γ,γ-dicarbethoxybutyric aldehyde 2,4-difluorophenyl hydrazone by boiling in 8% H_2SO_4. Synthesis of 5,7-difluoroTrp is described in detail in [10].

The structures of all substituted analogues of Trp and intermediate compounds were proven by elementary analysis, and IR and UV spectrometry.

Isolation of L-6-fluoroTrp from the racemic mixture of L- and D-6-fluoroTrp was carried out by using D-amino acid oxidase as described for L-Trp [11] with some modifications. A lesser amount, by two orders of magnitude, of the initial substance was used. The yield of the L-Trp analogue was 52%. Optical purity of the L-6-fluoroTrp preparation obtained was checked by testing the substrate activity in the reaction catalysed by D-amino acid oxidase (cf. [10]).

2.3. The degree of ATP–[^{32}P]PP_i exchange

The degree of ATP–[^{32}P]PP_i exchange was measured by the method [12] with minor modifications. Since BSA specifically binds L-Trp [13], a

Table 1
Interaction of tryptophan and its fluorinated derivatives with tryptophanyl tRNA synthetase in ATP–PP_i exchange reaction

Compound	K_M (app.), M	K_i, M	V_{max}*
L-Tryptophan	$(1.4 \pm 0.2) \times 10^{-7}$	–	1 ± 0.09
D,L-Tryptophan	$(1.5 \pm 0.2) \times 10^{-7}$	–	1 ± 0.1
D,L-4-Fluorotryptophan	$(2.9 \pm 0.5) \times 10^{-7}$	–	0.35 ± 0.07
D,L-5-Fluorotryptophan	$(1.7 \pm 0.3) \times 10^{-6}$	–	0.12 ± 0.01
L-6-Fluorotryptophan	$(2.9 \pm 0.5) \times 10^{-6}$	–	0.42 ± 0.03
D,L-6-Fluorotryptophan	$(3.2 \pm 0.6) \times 10^{-6}$	–	0.42 ± 0.06
D,L-7-Fluorotryptophan	$(1.0 \pm 0.1) \times 10^{-5}$	–	1.08 ± 0.1
D,L-5,7-Difluorotryptophan	–	$(2.0 \pm 0.5) \times 10^{-5}$	–
D,L-4,5,6,7-Tetrafluorotryptophan	–	$(1.2 \pm 0.3) \times 10^{-5}$	–

Each of the constants presented is a mean value from not less than three experiments. For reaction conditions see legend to fig. 1. When using D,L-mixtures, the concentrations of the L-form was assumed to be half of the concentration of D,L-amino acid added.

* With reference to value for L-tryptophan which was arbitrarily set equal to 1.0.

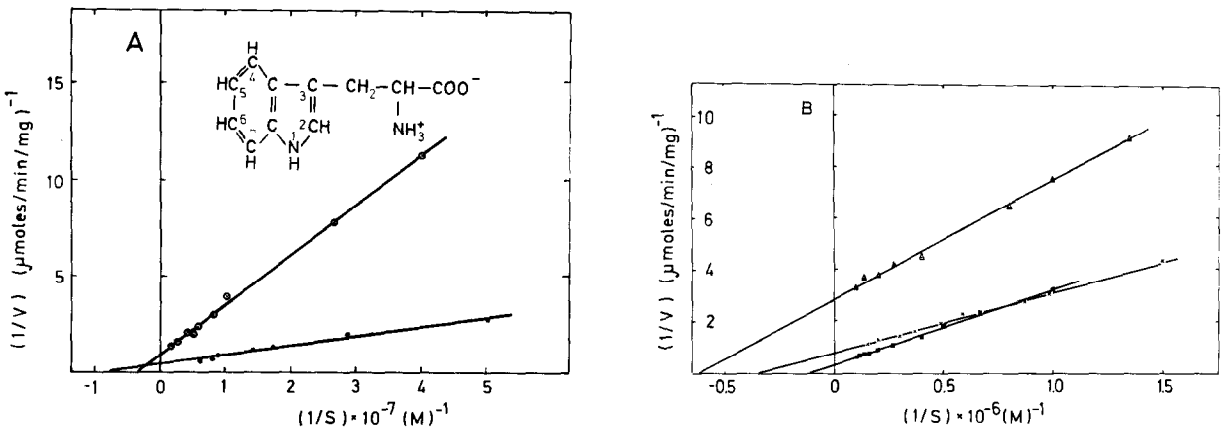

Fig. 1. Lineweaver–Burk plot for the rate of ATP–[^{32}P]PP_i exchange as a function of the concentration of following L-amino acids: (A) Trp (●–●–●), 4-fluoroTrp (○–○–○); (B) 5-fluoroTrp (△–△–△), 6-fluoroTrp (×–×–×), 7-fluoroTrp (■–■–■). Reaction mixture (0.2 ml) contained: 10 mM ATP; 3 mM pyrophosphate (0.26 mCi/mM); 0.01 M $MgCl_2$; 0.1 mg/ml gelatine; 0.05 M Tris–HCl, pH 7.5; 8 × 10^{-3} mg/ml TRSase.

dialysed gelatine was added to the mixture as a stabilizing agent [14].

3. Results and discussion

The interaction between TRSase and fluorinated tryptophans was investigated in terms of Michaelis–Menten constants or inhibition constants and maximum ATP–[^{32}P]PP_i exchange rates. The value of K_M (app.) was assumed to be a criterion of the affinity of substrates for TRSase; similarity of the values of K_M (app.) and K_S for Trp found previously [15] is thought to make this assumption valid. The results are listed in table 1. The kinetic parameters of the exchange reactions for fluorotryptophans acting as substrates were evaluated from Lineweaver-Burk plots [16] (fig. 1). For the analogues inhibiting the exchange reaction, the competitive character of inhibition was shown (fig. 2A). K_i values were determined as described in [17] (cf. fig. 2B).

The following conclusions can be drawn from table 1: (i) All the monofluorinated tryptophans are substrates in the ATP–[^{32}P]PP_i exchange reaction catalysed by TRSase; (ii) The polyfluorinated analogues studied competitively inhibit the reaction; (iii) The differences between the K_M (app.) and V_{max} values found for L- and D,L-Trp as well as for L- and D,L-6-fluoroTrp lie within the limits of experimental error; (iv) No correlation is observed between the values of the maximal ATP-PP_i exchange rate and Michaelis constants [5]. The affinities of the monofluorinated tryptophans depend on position of the fluorine atom in the tryptophan benzene ring. It decreases by two orders of magnitude from 4-fluoro to 7-fluoroTrp. It can be seen that the affinities of 5,7-difluoroTrp and 4,5,6,7-tetrafluoroTrp do not differ considerably from that of 7-fluoroTrp. This indicates that the substitution in position 7 is critical for the ability to bind to TRSase [6]. The value of the V_{max} for 7-fluoroTrp appeared to be equal to that found for Trp. The corresponding values for 4-fluoroTrp and 6-fluoroTrp are half this amount. The most considerable drop of the maximal exchange rate was observed for 5-fluoroTrp (10% of the V_{max} for Trp). Hence, the step of catalytic conversion of the substrate is especially sensitive to substitution at the position 5 of Trp.

The detailed study of the interactions of different fluorinated tryptophan derivatives indicates that, as would be expected, the indole ring is directly involved in the interaction with TRSase. Moreover the most marked effects are observed when the fluorine atoms are located in positions 7 and 5. This allows one to suggest that an important role may be played by the nitrogen atom of the pyrrole ring since just those

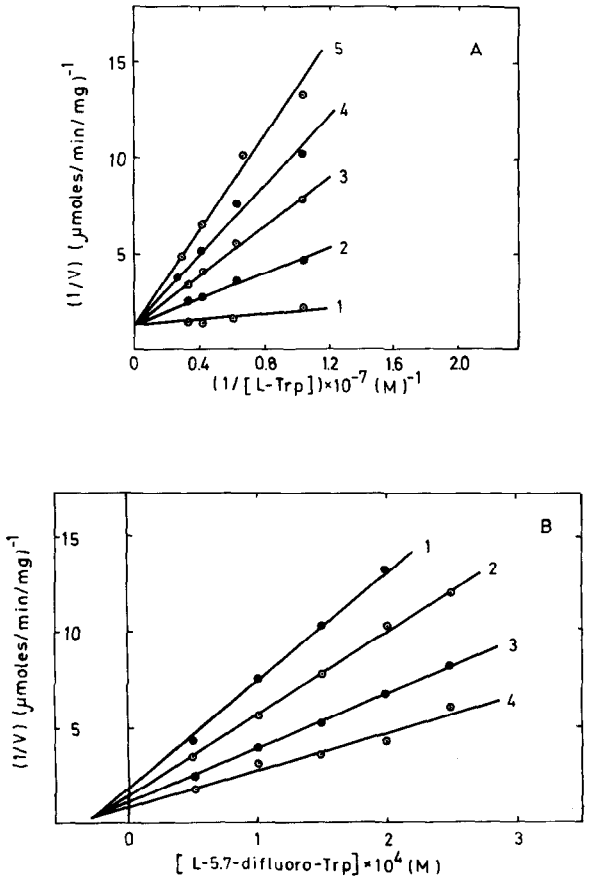

Fig. 2: (A) Lineweaver–Burk plot for the rate of ATP–[^{32}P]PP_i exchange as a function of the tryptophan concentration in the presence of different 5,7-difluoroTrp concentrations: (1) no inhibitor; (2) 0.05 mM; (3) 0.1 mM; (4) 0.15 mM; (5) 0.2 mM. (B) The reciprocal rate of ATP–[^{32}P]PP_i exchange as a function of the 5,7-difluoroTrp concentration in the presence of different Trp concentrations: (1) 0.095 μM; (2) 0.165 μM; (3) 0.245 μM; (4) 0.295 μM. For experimental details see legend to fig. 1. Similar plots were made for 4,5,6,7-tetrafluoro Trp.

fluorine atoms located in positions 5 and 7, viz. in *ortho* and *meta* positions to this nitrogen, affect its properties most strongly.

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