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THE ACTIVATION OF AMINO ACID ANALOGUES BY PHENYLALANYL- AND TYROSYL-*t*RNA SYNTHETASES FROM PLANTS

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Key Word Index—*Phaseolus aureus*; *Delonix regia*; *Caesalpinia tinctoria*; Leguminosae; Phe-*t*RNA synthetase; Tyr-*t*RNA synthetase; substrate specificity; amino acid analogues.

Abstract—Partially purified preparations of Phe- and Tyr-*t*RNA synthetases were obtained from seed or seedlings of *Phaseolus aureus*, *Delonix regia* and *Caesalpinia tinctoria*, and the ability of a variety of structural analogues of Phe or Tyr to act as alternative substrates or inhibitors was tested. 3-Hydroxymethylphenylalanine, a natural product of *C. tinctoria*, formed a particularly effective substrate for the Tyr-*t*RNA synthetase from *P. aureus*. The structural features commensurate with substrate activity in an analogue molecule are discussed.

INTRODUCTION

Phe- and Tyr-*t*RNA synthetases have been isolated from microbial [1–8], animal [9–12] and plant sources [13–15]. The general properties of the plant enzymes, especially their substrate specificities, have been studied using preparations from *P. aureus* [13], *Leucaena leucocephala* [13], and *Aesculus* spp. [14,15]. Phe-*t*RNA synthetases from various plant sources have been found to be less exacting in their amino acid substrate requirements than the corresponding Tyr-*t*RNA synthetases [13,15]; similar reports exist for some microbial systems [3,5,6]. The most detailed work with Phe-*t*RNA synthetase utilized an enzyme preparation from *A. hippocastanum* seed, and the influence on substrate activity was determined of (1) modifications in the side-chain, (2) substitutions on the phenyl ring, (3) replacement of phenyl by various heterocyclic ring systems, and (4) replacement of phenyl by unsaturated aliphatic branched-chain residues. Ring substitution by small non-ionizable atoms or groups was possible with retention of some substrate activity, and various alternative ring systems including the thienyl, pyridinyl and pyrazolyl groups were compatible with activity. 2-Amino-4-methylhex-4-enoic acid (R. alanine where R = MeCH=C(Me)–) also formed an excellent substrate. However few alterations of the alanine side chain were possible with retention of activity: compounds with either C_2 or C_4 side chains showed no substrate activity.

More recently we have had an opportunity to assess the possible substrate role of a further range of compounds showing structural affinities to either phenylalanine or tyrosine. Two of the compounds, 3-hydroxymethylphenylalanine (**1**) and 4-hydroxy-3-hydroxymethylphenylalanine (**2**), are natural products isolated from seed of *Caesalpinia tinctoria* [16], and the comparison of enzymes from different plant sources has been extended to include preparations of Phe- and Tyr-*t*RNA synthetases from this legume species.

RESULTS AND DISCUSSION

The compounds now tested as possible substrates for the two types of enzyme are illustrated.

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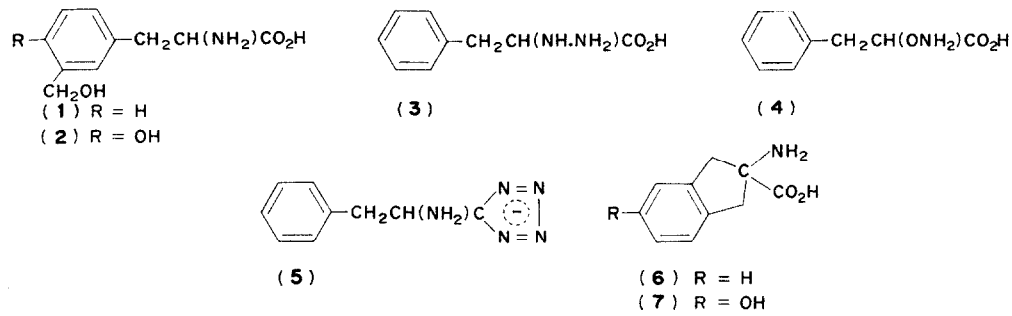

1 and **2** are ring-substituted phenylalanines, in which the larger hydroxymethyl group replaces a proton on the ring C-3 atom. Compounds **3** and **4** (α-hydrazino- and α-aminoxy-β-phenylpropionic acids, respectively) have larger basic groups replacing the normal α-amino group of phenylalanine, whilst in compound **5** a negatively charged tetrazole residue replaces the carboxyl group. The indanes, **6** and **7**, contain the essential features of phenylalanine and tyrosine respectively, but the functional amino and carboxyl groups are constrained in position by the additional methylene group bridging the normal α-carbon atom to the phenyl ring.

The activation of compounds **1** and **2** was tested in systems containing preparations of Phe-*t*NA synthetase from *C. tinctoria* and *P. aureus* (obtained by method A, see Experimental) and also with Tyr-*t*RNA synthetase from the same species (again from *P. aureus* by method A). The results are shown in Table 1: substrate activities, measured by the ATP–^{32}PPi exchange method, are reported for the two analogues as percentages of the rates of activation determined for the normal substrates, phenylalanine and tyrosine. The data present certain unusual features, especially the conclusion that Phe-*t*RNA synthetase from *C. tinctoria* accepts both **1** and **2** as substrates more readily than the comparable enzyme from *P. aureus*: this finding contrasts with the more usual observation that synthetases generally either do not activate, or activate relatively inefficiently, analogue amino acids that are constituents of the species yielding the enzyme preparation. The slight activation of tyrosine by Phe-*t*RNA synthetase from *Caesalpinia* might represent some residual contamination by Tyr-*t*RNA synthetase (although the latter enzyme was unstable during the prolonged dialysis included in the fractionation scheme), but the activations of **1** and **2** cannot be ascribed to similar contamination. The partially purified Tyr-*t*RNA synthetase preparations showed no affinity for **2**, but **1** was activated by enzyme from both *Phaseolus* and *Caesalpinia*, especially efficiently by the former. Molecular models show that the hydroxyl-proton of the 3-hydroxymethyl group of **1** can attain a position, relative to the remainder of the molecule, almost identical to that of the phenolic-H of tyrosine. Presumably, the proton of tyrosine becomes involved

Table 1. The ability of ring-substituted phenylalanine derivates to act as substrates for Phe- and Tyr-*t*RNA synthetases

Substrate	*P. aureus* enzyme*	*C. tinctoria* enzyme
A. Phe-*t*RNA synthetase: v as $^{0}/_{0}$ V_{max} (phe)		
L-Phenylalanine (10 mM)	100	100
L-Tyrosine (10 mM)	0	19
L-3-Hydroxymethylphenylalanine (20 mM)	13	69
L-4-Hydroxy-3-hydroxymethylphenylalanine (20 mM)	3	13
B. Tyr-*t*RNA synthetase: v as $^{0}/_{0}$ V_{max} (tyr)		
L-Phenylalanine (10 mM)	0	0
L-Tyrosine (10 mM)	100	100
L-3-Hydroxymethylphenylalanine (20 mM)	0	0
L-4-Hydroxy-3-hydroxymethylphenylalanine (20 mM)	142	29

* Enzymes prepared by method A (see Experimental).

in transient ligand bonding at the active site of Tyr-*t*RNA synthetase during activation, and the stereochemical disposition of the 3-hydroxymethyl may permit similar bonding through its hydroxyl-H atom, although in other circumstances the behaviour of aliphatic and aromatic hydroxyls appear very different.

Compounds **3**–**6** were tested as possible substrates or inhibitors of Phe-*t*RNA synthetase from *P. aureus* (obtained by method B). α-Hydrazino-β-phenylpropionic acid (**3**), alone among this group, exhibited some substrate activity, being activated (at enzyme-saturating concentrations) at 15% of the rate measured with phenylalanine. K_m values for the normal substrate and the analogue were calculated at $1{\cdot}6 \times 10^{-5}$ M and $\sim 7 \times 10^{-4}$ M, respectively. Slight competition between the two compounds for binding at the enzyme's active site was suggested by rate measurements: the rates of ATP–^{32}PPi exchange in the presence of a mixture of the analogue (40 mM) and phenylalanine (0·125 mM) was only 98% of that determined with phenylalanine alone. Although the tetrazole derivative of phenylalanine (**5**) did not behave as a substrate, it showed inhibitory activity and exhibited a K_i value of 3×10^{-3} M for the Phe-synthetase from *P. aureus*. A similar value ($2{\cdot}3 \times 10^{-3}$ M) was measured for an enzyme preparation from *D. regia* [16], which had K_m(phe) $6{\cdot}1 \times 10^{-6}$ M. The tetrazole derivative of phenylalanine also acted as a weak inhibitor of a Tyr-*t*RNA synthetase preparation from *P. aureus*, having a K_i value of 5×10^{-2} M [K_m(tyr) $= 8 \times 10^{-5}$ M].

The observation that an α-hydrazino group can replace the α-amino group of phenylalanine with retention of some substrate potential indicates that its slightly increased size and basicity still permit the adjacent carboxyl group to attain an orientation in which activation is possible by binding to the α-phosphate group of ATP. By contrast, substitution by an even larger, but less basic α-aminoxy group leads to a compound (**4**) showing no affinity for the Phe-*t*RNA synthetase. The reduced basicity may be the more important factor in preventing the carboxyl group attaining the required conformation for binding. The constraints imposed upon both the amino and carboxyl groups by the bridging methylene in the indane derivative (**6**) also lead, not unexpectedly, to complete loss of affinity for the enzyme.

2-Amino-2-carboxy-6-hydroxyindane (**7**) was tested as a possible substrate for the Tyr-*t*RNA synthetase prepared from *P. aureus* (see above): the hydroxy groups in **7** and in tyrosine exhibit a similar stereochemical relationship to the remainder of their respective molecules. The Tyr-synthetase has a K_m(tyr) of approx. 8×10^{-5} M; **7** exhibited some substrate activity, with V_{max} equal to 15% of that determined for tyrosine and $K_m \sim 10^{-3}$ M. Obviously, the constraints imposed on the positioning of the carboxyl group are less restrictive for the binding of indane derivatives to the Tyr-enzyme than to the Phe-synthetase. Certain other evidence suggests that carboxyl group orientation may not be a critical factor for the formation of tyr-adenylate: for instance, the bacterial enzyme can activate D-tyr as well as the L-isomer [17].

The studies with these additional analogues have extended the range of possible substitutions within the phenylalanine or tyrosine molecule known to be still compatible with the retention of some affinity for the corresponding aminoacyl-*t*RNA synthetases. The results then provide further support for the idea that the specificity of these two enzymes, for at least the amino acid substrate, is less rigorous than that of most other synthetases.

EXPERIMENTAL

Plant materials. Seeds of *P. aureus* were purchased from the Bombay Emporium, London. *D. regia* seed was the gift of Prof. L. B. Thrower (Hong Kong) and *C. tinctoria* was kindly provided by Dr. E. Bornemisza (Lima, Peru).

Amino acid analogues. 3-Hydroxymethylphenylalanine (**1**) and 4-hydroxy-3-hydroxymethylphenylalanine (**2**) are natural products isolated from seed of *C. tinctoria* [18]. α-Hydrazino-β-phenylpyruvic acid (**3**), α-aminoxy-β-phenylpyruvic acid (**4**) and the tetrazole analogue of phenylalanine (**5**) were gifts of Dr. J. S. Morley (ICI Pharmaceutical Division, Macclesfield, Cheshire). The two aminoindane carboxylic acids (**6** and **7**) were gifts of Dr. R. M. Pinder (Porton Down, Salisbury, Wilts.). Compounds **1** and **2** were L-isomers, whilst **3**–**7** were DL-racemates.

Radiosotopic chemicals. ^{32}P-Pyrophosphate was prepared as previously described [19] to give a product of sp. act. 0·3–0·5 μCi/μmol.

Determination of protein. During enzyme fractionations, protein was determined by the Lowry method [20] or from extinction values measured at 260 and 280 nm [21] with bovine serum albumin as a standard.

Fractionation of aminoacyl-tRNA synthetases. (i) Enzymes from *P. aureus*. Seed meal was used as a source of enzymes. In method A, the meal (20 g) was extracted by grinding with 200 ml of extractant buffer (containing 0·2 M Tris–HCl, pH 8·0, 15% (w/v) glycerol, 20 mM $MgCl_2$ and 25 mM mercaptoethanol). The clarified extract was subjected to $(NH_4)_2SO_4$ fractionation; the 65–75% saturation cut was retained for further purification of the Phe- and Tyr-synthetases. Phe-synthetase

was obtained by applying the redissolved $(NH_4)_2SO_4$ ppt. (now in 0·05 M Tris–HCl buffer) after dialysis to a DEAE-cellulose column, equilibrated in 0·02 M buffer. The column was eluted with 0·02 M buffer containing an increasing gradient of KCl concns. The 3 fractions having high Phe-*t*RNA synthetase activity were pooled and used in the activation experiments. Tyr-synthetase was unstable during fractionation on the DEAE-cellulose column, but could be obtained by acetone fractionation of the redissolved $(NH_4)_2SO_4$ fraction: the enzyme was conc in the 33–50% (v/v) acetone fraction. In method B, a more highly purified Phe-*t*RNA synthetase was obtained by introducing additional steps into the purification procedure after the initial precipitation by $(NH_4)_2SO_4$. The redissolved ppt. was subjected to a heat treatment (64°, 15 min, in presence of 2 mM ATP and 0·4 mM phe) [22], which partially inactivated contaminating aminoacyl-*t*RNA synthetases. Acetone fractionation (18–24% cut) and DEAE-cellulose treatment followed giving an enzyme showing a 78-fold increase in sp. act. in comparison with the original extract. The Tyr-synthetase was prepared by method B again involving a heat denaturation at 57° for 15 min in the presence of ATP (2 mM) and tyr (2 mM). Acetone and $(NH_4)_2SO_4$ fractionations formed other steps in the procedure leading to a 14-fold purification. (ii) Enzymes from *C. tinctoria*. Seeds, from which testa material had been removed, were used as a source of Phe-enzyme. The powdered material (4·2 g) was extracted with 100 ml extractant (containing buffer and additives as above, plus 0·3% polyvinylpyrrolidone, MW 44000). From the extract, clarified by centrifuging, a 60–75% saturated $(NH_4)_2SO_4$ fraction was obtained. This contained Phe-*t*RNA synthetase, and some Tyr-synthetase: the latter enzyme was largely lost during prolonged dialysis of the redissolved $(NH_4)_2SO_4$ fraction against 0·02 M buffer, and the dialysate was used as the Phe-*t*RNA synthetase preparation in the activation experiments. Tyr-*t*RNA synthetase was prepared from 7-day-old seedlings. Proteins present in the seedling extract were completely precipitated by saturating with $(NH_4)_2SO_4$. The redissolved proteins then were subjected to $(NH_4)_2SO_4$ fractionation to give a 55–75% saturation cut. This contained Tyr-synthetase, and some Phe-synthetase activity, but if the preparation was stored for 4 days at −20° in the presence of tyr (0·4 mM), ATP (2 mM) and glycerol (30%), all Phe-synthetase activity was lost. The remaining soln was used as the Tyr-*t*RNA synthetase in the activation experiments. (iii) Phe-*t*RNA synthetase from *D. regia*. The enzyme was purified from a seed meal extract by steps similar to those used with *P. aureus* (method B). The extraction buffer contained 10% glycerol and 40 mM mercaptoethanol and the additives were present during all later stages of the fractionation procedure. The sequential purification steps applied to the extract were $(NH_4)_2SO_4$ fractionation, heat treatment (64° for 15 min in presence of 2 mM ATP and 0·4 mM phe), acetone fractionation and DEAE-cellulose chromatography. A 76-fold enhancement of sp. act. was effected.

ATP–^{32}PPi exchange assay procedure. Incubation mixture normally contained: Tris–HCl buffer pH 8·0 980–100 μmol), $MgCl_2$ (10 μmol), ATP (2 μmol), ^{32}PPi (2 μmol), amino acid (10 μmol of L-form; higher concns were employed in some experiments studying K_m or K_i values which refer to L-forms) and enzyme preparation: total vol. 1 ml. Reaction was performed at 37° usually for 15 min and terminated by addition of 5% (w/v) TCA. Labelled ATP was separated from unchanged ^{32}PPi by absorbing the nucleotise triphosphate on to charcoal [23], and other conditions were as previously described [24].

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