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Tyrosyl Transfer Ribonucleic Acid Synthetase from *Escherichia coli* B. Analysis of Tyrosine and Adenosine 5′-Triphosphate Binding Sites†

Daniel V. Santi* and Van A. Peña

Department of Pharmaceutical Chemistry and Department of Biochemistry and Biophysics, University of California, San Francisco, California 94122. *Received September 25, 1972*

Structural and stereochemical requirements for substrate binding to tyrosyl-tRNA synthetase from *Escherichia coli* B have been investigated using analogs of L-tyrosine and ATP. The two major binding loci for the amino acid have been shown to be the phenol and amine moieties. The phenolic hydroxyl is bound as its neutral form and does not act as a hydrogen bond acceptor. It is the primary site of recognition and its omission results in at least a 10,000-fold loss in binding. The amino group of the substrate binds as its protonated form to an area of the enzyme which is probably best represented as anionic. The carboxylate moiety does not appear to be a contact point and may be substituted by disparate groups with little effect on binding. Adjacent to the carboxylate binding site lies a hydrophobic region and a group capable of interaction with negatively charged substituents. The stereospecificity of the enzyme is not exact and D enantiomers complex with only small losses in affinity. These losses may be attributed to the energy required for rotation about the C_α–C_β bond of D-tyrosine and its analogs or an analogous conformational change of the enzyme, which is necessary to accommodate the major binding loci. Binding of ATP requires interactions of the intact triphosphate moiety. Analogs not possessing this moiety bind as weak, noncompetitive inhibitors and may interact as dimers at a site remote from that which binds to ATP.

Each of the aa-tRNA‡ synthetases is responsible for the attachment of an amino acid to the tRNA's which recognize the codons for that amino acid. Should an uncorrected mistake occur at this stage, the amino acid would be incorporated into an incorrect position of the protein.[1] The overall reaction catalyzed by these enzymes appears to involve (1) activation of a particular amino acid to form an aminoacyl adenylate intermediate and (2) transfer of the activated amino acid to its cognate tRNA.

$$\text{aa} + \text{ATP} \rightleftharpoons \text{aa-AMP} + \text{PP}_i \quad (1)$$

$$\text{aa-AMP} + \text{tRNA} \rightleftharpoons \text{aa-tRNA} + \text{AMP} \quad (2)$$

An understanding of the molecular basis for the specificity of these enzymes requires a knowledge of the intermolecular forces which lead to substrate recognition and binding, as well as differences in the binding sites among the various synthetases.

A number of studies have recently been reported in which competitive inhibitors have been utilized to map the active sites of these enzymes[2-5] in an attempt to answer pertinent questions regarding their specificity. Investigations of this type also provide fundamental information necessary for the design of potent and specific inhibitors of these enzymes which should be useful for a variety of biological investigations.

In the present work, analogs of L-tyrosine and ATP are utilized to probe the topography and localized environment of the substrate binding sites of tyrosyl-tRNA synthetase from *Escherichia coli* B. Comparisons with other activating enzymes from the same source have permitted the assignment of a number of differences and similarities in substrate binding and recognition sites.

Materials and Methods

A 550-fold purified preparation of TRS was isolated from *E. coli* B harvested in the late log phase (General Biochemicals) by the method of Calendar and Berg[6] with the exception that C-γ gel fractionation was omitted. Under standard assay conditions with saturating amounts of substrates, this preparation catalyzed the exchange of 55 μmol of $^{32}PP_i$ into ATP per minute per milligram of protein. Inhibition constants (K_i) were obtained by double-reciprocal plots[7] varying L-tyrosine or ATP·Mg in equal increments from 30 to 3.3 nmol/ml or 1.25 to 0.14 μmol/ml, respectively; all other components were held constant at the concentrations given above. Values for K_i for noncompetitive inhibitors were calculated using equations presented by Dixon and Webb.[8] The $MgCl_2$ concentration used falls on the broad optimum (5-15 m*M*) where the exchange rate is insensitive to small variations. When inhibitors known to complex with Mg^{2+} were tested, an equivalent amount of $MgCl_2$ was added to ensure against depletion. Protein concentration was determined spectrophotometrically by the method of Warburg and Christian[9] or Groves, *et al.*[10] One unit of enzyme is that amount incorporating 1 μmol of $^{32}PP_i$ into ATP in 1 min in the standard assay. The ATP-PP_i exchange assay was carried out as described by Calendar and Berg.[6] The standard assay reaction mixture (1 ml) contained 100 m*M* Na cacodylate (pH 7.0), 5 m*M* $MgCl_2$, 2 m*M* ATP, 2 m*M* $Na_4P_2O_7$ (*ca.* 10^5 cpm/μmol), 10 m*M* 2-mercaptoethanol, 0.1 mg of BSA, 0.1 m*M* tyrosine, and enzyme (0.005–0.007 unit). The assay mixture was incubated at 37° and, at appropriate

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‡Abbreviations used are: TRS, tyrosyl-tRNA synthetase (E.C. 6.1.1.1) of *Escherichia coli* B; PRS, phenylalanyl-tRNA synthetase (E.C. 6.1.1.4) of *Escherichia coli* B; $tRNA^{Tyr}$, tRNA specific for tyrosine acceptance; aa-tRNA, aminoacyl-tRNA; aa-AMP, aminoacyl adenylate; α,β-CH_2-ATP, α,β-methylene ATP.

times, quenched with 0.7 ml of 15% $HClO_4$ which contained 0.4 *M* PP_i. The ATP was adsorbed on Norit and collected on Whatman GF/C glass filters, and radioactivity was counted on a Nuclear Chicago planchet counter.

L-Methyl (**5**), L-ethyl (**13**), and L-benzyl (**19**) tyrosinate hydrochlorides, L-tyrosine amide (**7**), L-tyrosine hydroxamate (**8**), L-tyrosylglycine (**34**), tyramine·HCl (**2**), *p*-hydroxyphenylpropionic (**42**), *p*-hydroxyphenyllactic (**43**), and *p*-hydroxyphenylpyruvic (**44**) acids, L-phenylalanine (**51**), D-tyrosine, AMP (**61**), 3′,5′-cyclic AMP (**62**), and 3′-deoxyadenosine (**72**) were products of Sigma Chemical Co. Candicine iodide (**48**), ATP (**57**), ADP (**60**), adenosine (**71**), and adenine (**80**) were obtained from Aldrich Chemical Co. L-Tyrosyl-L-glutamic acid (**40**) and α,β-CH_2-ATP (**59**) were obtained from Miles Laboratories; other peptides, as well as L-tyrosine-β-napthylamide·HBr (**33**) and dopamine·HCl (**55**), were obtained from Nutritional Biochemical Corp. The amphetamine analogs listed in Tables IV and V were gifts from Smith, Kline and French Laboratories. *cis*-4-(6-Amino-9*H*-purin-9-yl)-*trans*-2-*trans*-3-dihydroxycyclopentanemethanol (**73**) was supplied by Southern Research Institute, and 1-amino-*p*-hydroxyphenylphosphonic acid (**10**) was obtained from Calbiochem. 2,3,5,6-Tetrafluoro-DL-tyrosine (**56**) was a gift from R. Filler. The D-methyl (**6**) [mp 189–190°, $[\alpha]^{25}D$ 75.3° (*c* 1, pyridine)], L-*n*-propyl (**14**) (mp 148–149°, lit.[11] mp 148°), L-*n*-butyl (**15**) (mp 165–166°, lit.[12] mp 162–164°), L-*sec*-butyl (**16**) (mp 175–176°, lit.[13] mp 172°), and the L-isoamyl (**18**) (mp 160–162°, lit.[11] mp 158°) tyrosinates were prepared by the general method of Boissonnas.[14] *tert*-Butyl tyrosinate (**17**) was prepared as described by Roeskie.[15] *p*-Hydroxybenzylamine·HCl (**49**) (mp 195–196°, lit.[16] mp 195°) was prepared by the general method of Freifelder and Ng,[17] and *p*-hydroxyphenylpropylamine·HCl (**50**) (mp 160–161°, lit.[18] mp 158–159°) was obtained by $LiAlH_4$ reduction of *p*-hydroxyphenylpropionamide employing the general synthesis of Schoenenberger, *et al.*[19] D-Tyrosinol·HCl (**4**) [mp 165–166°, $[\alpha]^{25}D$ 18.9 (*c* 2.5, H_2O)] was prepared by $LiAlH_4$ reduction[20] of the D-methyl ester (**6**). Both L- and D-tyrosinol adenylate (**11**, **12**), $[\alpha]^{25}D$ −3.47° (*c* 3.0, H_2O) and $[\alpha]^{25}D$ +2.86° (*c* 1.5, H_2O), respectively, were prepared according to the literature procedure for the L isomer[21] except that *N*-carbobenzoxy-D-tyrosinol and *N*-carbobenzoxy-L-tyrosinol prepared from the respective *N*-carbobenzoxy tyrosines using the general procedure of Ishizumi, *et al.*,[22] were employed instead of the *N,O*-dicarbobenzoxytyrosinols. Both *cis*-9-(4-hydroxymethylcyclohexyl)adenine (**75**) and *cis*-9-(3-hydroxymethylcyclopentyl)adenine (**74**) were prepared as described by Schaeffer, *et al.*[23] (see Chemistry). 6-Amino-9-(5-hydroxypentyl)purine (**78**) (mp 192–193°; HCl salt, mp 168–171°, lit.[24] mp 164–168°) and 9-(5-hydroxyhexyl)adenine (**79**) (mp 196–197°. *Anal.* ($C_{11}H_{17}N_5O$) C, H, N. Lit.[25] mp 184–187°) were prepared from adenine using a direct alkylation procedure reported by Montgomery and Thomas.[26] The adenosine 5′-alkylphosphonates were gifts of K. A. Montgomery of this laboratory.

Experimental Section

Melting points were taken in capillary tubes on a Mel-Temp block and are corrected. All analytical samples had proper ir spectra and moved as a single spot on tlc with Brinkmann silica gel GF; each gave combustion values for C, H, and N within 0.4% of theoretical values. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. Specific rotations were obtained using a Perkin-Elmer 141 polarimeter.

The L-tyrosine amides were prepared by the following general procedures.

***N*-Carbobenzoxy-L-tyrosylanilide (95).** Isobutyl chloroformate (1.37 g, 10 mmol) in THF (5 ml) was added to a stirred, ice-cold solution of *N*-carbobenzoxy-L-tyrosine (3.15 g, 10 mmol) and Et_3N (1.02 g, 10 mmol) in THF (20 ml). After 5 min, freshly distilled aniline (930 mg, 10 mmol) in THF (5 ml) was added, and the solvent was removed at reduced pressure after 10 min. The residue was dissolved in $CHCl_3$ (25 ml), extracted with H_2O (2 × 10 ml), and dried ($MgSO_4$). After concentrating this solution to 5 ml, C_6H_6 (50 ml) was added while heating the mixture. On cooling, the precipitated gel was filtered to give 1.98 g (50.7%) of white powder, mp 188–188.5°. Two additional precipitations from $CHCl_3$–C_6H_6 gave the analytical sample, mp 188–188.5°. *Anal.* ($C_{23}H_{22}N_2O_4$) C, H, N.

L-Tyrosine Ethylamide Hydrobromide (21). A solution of 84 (800 mg, 2.34 mmol) in HOAc (5 ml) was added to a solution of phenol (10 g) in 36% HBr–HOAc (10 ml). After 1 hr at ambient temperature the solution was concentrated to 5 ml and anhydrous Et_2O (200 ml) was added. The precipitate was triturated with Et_2O to remove occluded phenol, dissolved in H_2O (5 ml), and decolorized with Norit for 10 min with heating on a steam bath. The solvent was removed at reduced pressure to give 631 mg (93%) of crude HBr salt, which was recrystallized twice from MeOH–PrOH to give 520 mg (77%) of the analytical sample, mp 164–166°. *Anal.* ($C_{11}H_{17}BrN_2O_2$) C, H, N.

In cases where the HBr salts could not be readily crystallized, isolations were performed by one of the following methods.

(A) Free Base. L-Tyrosine Benzylamide (25). The crude HBr salt (627 mg) was made basic with 5% $NaHCO_3$ (5 ml), extracted with $CHCl_3$ (4 × 10 ml), and dried ($MgSO_4$). This solution was evaporated to dryness at reduced pressure to give 274 mg (48.4%) of white powder which was recrystallized (twice) from toluene–$CHCl_3$ to yield 261 mg (46%) of the analytical sample, mp 175.1–175.5°. *Anal.* ($C_{16}H_{18}N_2O_2$) C, H, N.

(B) *p*-Toluenesulfonate Salt. L-Tyrosylanilide *p*-Toluenesulfonate (32). The crude HBr salt (540 mg) was dissolved in H_2O containing 2 equiv of TsOH·H_2O (615 mg). The granular salt precipitated with gradual cooling to give 665 mg (76%) of crude product, mp 265–267°. A portion of this material was recrystallized (three times) from MeOH–H_2O to give the analytical sample, mp 266–267°. *Anal.* ($C_{22}H_{24}N_2O_5S$) C, H, N.

(C) Acetate Salt. L-Tyrosine Phenylpentylamide Acetate (31). The crude HBr salt (500 mg) was passed through a column (1.1 × 20 cm) of Dowex 1-X8 (200–400 mesh), acetate form. After lyophilization, the acetate salt (420 mg) was eluted from a column (1.5 × 25 cm) of Amberlite IRC-50 (50–100 mesh), H^+ form, with a linear gradient from 0 to 10% v/v HOAc–H_2O (400 ml total volume). Lypohilization of fractions containing product gave 110 mg (23%) of the analytical sample, mp 86–87°. *Anal.* ($C_{22}H_{30}N_2O_4$) C, H, N.

(D) HCl Salt. L-Tyrosine Propylamide Hydrochloride (22). Th The crude HBr salt (390 mg) was converted to the free base (method A) and treated with 0.1 *N* HCl (5 ml). Lyophilization of this solutior gave 75 mg (21.1%) of the analytical sample (hygroscopic solid), mp 70–75°. *Anal.* ($C_{12}H_{18}ClN_2O_2$) C, H, N.

6-Amino-9-(3-hydroxypropyl)purine (76). A suspension of adenine (2.0 g, 14.3 mmol) and NaH (50% dispersion in mineral oil) (0.70 g, 14.5 mmol) in dry DMF (25 ml) was magnetically stirred (protected from moisture) for 4 hr at ambient temperature. A solution of 1-bromo-3-hydroxypropane (2.07 g, 14.9 mmol) in dry DMF (20 ml) was added dropwise to the suspension. After the mixture clarified (*ca.* 20 hr) the solvent was removed at reduced pressure and the residue recrystallized from EtOH to give 1.72 g (62%) of white crystals, mp 209–210° (lit.[27] mp 204–206°).

6-Amino-9-(4-hydroxybutyl)purine (77). A solution of 4-chlorc butyl acetate (5.42 g, 36 mmol) in dry DMF (50 ml) containing KI (100 mg, 0.6 mmol) was added to an equivalent amount of Na adenide (prepared as described for **76**). The mixture was stirred at 50° and protected from moisture until the solution clarified (*ca.* 26 hr). After cooling, the mixture was evaporated to dryness, and the crystalline residue was extracted with Me_2CO and filtered. Removal of the solvent at reduced pressure gave 2.76 g (30.8%) of crude 6-amino-9-(4′-acetoxybutyl)purine, mp 164–164.5° (lit.[28] mp 161°), which was dissolved in MeOH saturated with NH_3 at 0° (100 ml). After 72 hr, the solvent was evaporated and the residue recrystallized (twice) from EtOH to give 2.42 g (26.9%) of white crystals, mp 197–198.5° (lit.[27] mp 196–197°).

***N*-Carbobenzoxy-L-tyrosinol.** Isobutyl chloroformate (1.37 g, 10 mmol) in dry THF (5 ml) was added to a magnetically stirred, ice-cold solution of *N*-carbobenzoxy-L-tyrosine (3.15 g, 10 mmol) and Et_3N (1.02 g, 10 mmol) in THF (25 ml). After 5 min the reaction mixture was filtered through Celite into a stirred suspension

of $NaBH_4$ (757 mg, 20.0 mmol) in THF (25 ml). After stirring at 0° for 1 hr, cold HCl (2.5 *M*, 30 ml) was slowly added to the mixture, and the solvent was removed *in vacuo* at ambient temperature. The residue (5.3 g) was dissolved in $CHCl_3$ (50 ml), extracted with H_2O (2 × 20 ml), and dried ($MgSO_4$). After concentrating to 10 ml, C_6H_6 (50 ml) was added while heating the mixture. The product crystallized on standing as hard nodes to give 2.58 g (86%) of material suitable for further transformation, mp 91–92°. A portion (200 mg) was purified on a silica gel column (1.5 × 25 cm) which was eluted with $CHCl_3$–THF (4/1 v/v). A total of 68 fractions (6 ml, 1 ml/min) were taken, and material in fractions 18–30 was collected and crystallized from $CHCl_3$–C_6H_6 to give 180 mg (90%) of the analytical sample: mp 92–93°; $[\alpha]^{25}D$ −31.25° (*c* 3.4, MeOH). *Anal.* ($C_{17}H_{19}NO_4$) C, H, N.

N-Carbobenzoxy-D-tyrosinol. This compound was prepared on a 1.5-mmol scale using the procedure for the L isomer: yield 353 mg (78%) from $CHCl_3$–C_6H_6; mp 91–92°; $[\alpha]^{25}D$ +30.06° (*c* 3.5, MeOH).

Chemistry

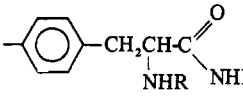
The synthetic route initially attempted for synthesis of the the L-tyrosine amides **20–32** involved mixed anhydride condensation[29] of the readily available *N,O*-dicarbobenzoxy-L-tyrosine.[30] Although this reaction proceeded well, removal of blocking groups by reported methods led to an intractable mixture of products. Utilization of the *N*-carbobenzoxy-L-tyrosine amides resulted in a significant reduction of byproducts upon blocking group removal with HBr–HOAc, refluxing TFA, or H_2/Pt, but impurities were present in amounts which encumbered purification. As reported for TFA removal of Cbz groups,[31] addition of phenol to HBr–HOAc resulted in a great reduction of side products and permitted isolation of the tyrosine amides in reasonable yields (Table I).

The 9-(ω-hydroxyalkyl)adenines **76–79** were prepared in good yield by direct alkylation of sodium adenide in DMF with the ω-halohydrins or, where base-catalyzed cyclization was likely with the halohydrins, their *O*-acetates. The position of alkylation was verified spectrophotometrically.[32,33]

The modifications employed in the preparation of *cis*-9-(3-hydroxymethylcyclopentyl)adenine (**74**)[23] resulted in significantly higher yields and are noteworthy. 3-Oxocyclopentanecarboxylic acid, a key intermediate, was prepared in excellent yield by the method of Shemyakin, *et al.*,[34] rather than the referenced procedure of Arendaruk, *et al.*,[35] which proceeded poorly in our hands. Although conversion of this compound to the corresponding oxime was reported to proceed in only 20% yield,[36] a quantitative yield was readily obtained using the general procedure of Schneider and Dillman.[37] The cyclization of *cis*-3-(5-amino-6-chloro-4-pyrimidinylamino)cyclopentylcarbinol to *cis*-3-(6-chloro-9-purinyl)cyclopentylcarbinol at ambient temperature afforded a 50% higher yield than the reported method.[38]

Enzyme Results

Inhibitory properties of a number of carboxyl-substituted derivatives of tyrosine in the TRS-catalyzed ATP-PP_i exchange reaction are given in Table II. Substitution of carboxylate by such disparate groups as hydrogen (**2**), hydroxymethyl (**3**), carboxamido (**7**), hydroxamate (**8**), methyl (**9**), or phosphonate (**10**) results in inhibitors which are competitive with respect to tyrosine and bind 1.5- to 10-fold less effectively than tyrosine. Although the L-methyl ester **5** exhibits a 25-fold loss in binding, this result, as well as the inhibition data for esters listed elsewhere, should be regarded as upper limit approximations to the true K_i since they undergo hydrolysis (*ca.* 3%) during assay, and the tyrosine liberated leads to high blanks. The stereospecificity of TRS is not exact with respect to binding properties and the D isomers listed exhibit K_i

Table I. Physical Constants of L-HO– 

No.	R	R′	Salt	Yield, %	Mp, °C	Formula
20	H	$-CH_3$	HBr[a]	42	234–236	$C_{10}H_{15}BrN_2O_2$
21	H	$-CH_2CH_3$	HBr	77	164–166	$C_{11}H_{17}BrN_2O_2$
22	H	$-CH_2CH_2CH_3$	HCl[b]	21	70–75	$C_{12}H_{19}ClN_2O_2$
23	H	$-CH_2(CH_2)_2CH_3$	*c*	56	144–145	$C_{13}H_{20}N_2O_2$
24	H	$-CH_2(CH_2)_3CH_3$		59	144–146	$C_{14}H_{22}N_2O_2$
25	H	$-CH_2C_6H_5$		46	175–176	$C_{16}H_{18}N_2O_2$
26	H	$-CH_2(4\text{-}NO_2C_6H_4)$	HBr	62	175–178	$C_{16}H_{18}BrN_3O_4$
27	H	$-CH_2(4\text{-}CH_3C_6H_4)$	HBr	68	180–181	$C_{17}H_{21}BrN_2O_2$
28	H	$-CH_2CH_2C_6H_5$		70	68–69	$C_{17}H_{20}N_2O_2$
29	H	$-CH_2CH_2CH_2C_6H_5$		36	48–50	$C_{18}H_{22}N_2O_2$
30	H	$-CH_2(CH_2)_2CH_2C_6H_5$	2HOAc[d]	74	98–100	$C_{23}H_{32}N_2O_6$
31	H	$-CH_2(CH_2)_3CH_2C_6H_5$	HOAc[d]	23	86–87	$C_{22}H_{30}N_2O_4$
32	H	$-C_6H_5$	TsOH	76	265–267	$C_{22}H_{24}N_2O_5S$
83	Cbz	$-CH_3$		79	105–106	$C_{18}H_{20}N_2O_4$
84	Cbz	$-CH_2CH_3$		75	115–116	$C_{19}H_{22}N_2O_4$
85	Cbz	$-CH_2CH_2CH_3$		84	168–169	$C_{20}H_{24}N_2O_4$
86	Cbz	$-CH_2(CH_2)_2CH_3$		79	106–108	$C_{21}H_{26}N_2O_4$
87	Cbz	$-CH_2(CH_2)_3CH_3$		88	96–97	$C_{22}H_{28}N_2O_4$
88	Cbz	$-CH_2C_6H_5$		87	153–154	$C_{24}H_{24}N_2O_4$
89	Cbz	$-CH_2(4\text{-}NO_2C_6H_4)$		22	181–182	$C_{24}H_{23}N_3O_6$
90	Cbz	$-CH_2(4\text{-}CH_3C_6H_4)$		81	149–150	$C_{25}H_{26}N_2O_4$
91	Cbz	$-CH_2CH_2C_6H_5$		75	150–151	$C_{25}H_{26}N_2O_4$
92	Cbz	$-CH_2CH_2CH_2C_6H_5$		63	113–114	$C_{26}H_{28}N_2O_4$
93	Cbz	$-CH_2(CH_2)_2CH_2C_6H_5$		69	135–136	$C_{27}H_{30}N_2O_4$
94	Cbz	$-CH_2(CH_2)_3CH_3C_6H_5$		51	106–107	$C_{28}H_{32}N_2O_4 \cdot 0.5H_2O$
95	Cbz	$-C_6H_5$		51	188–189	$C_{23}H_{22}N_2O_4$

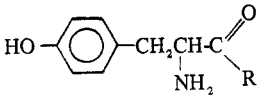
[a] All HBr salts recrystallized from MeOH-*n*-PrOH. [b] Lyophilized solid. [c] All free bases recrystallized from $CHCl_3$–C_6H_6. [d] Lyophilized solid.

Table II. Inhibitory Power of Carboxyl-Substituted Derivatives of Tyrosine

No.	Compound	K_i, m*M*
1	L-Tyrosine	0.0036 (K_m)[a]
2	Tyramine	0.0056[b]
3	L-Tyrosinol	0.016[c]
4	D-Tyrosinol	0.067[d]
5	L-Tyrosine methyl ester	0.09[e]
6	D-Tyrosine methyl ester	0.5[d]
7	L-Tyrosinamide	0.31[f]
8	L-Tyrosine hydroxamate	0.019
9	DL-*p*-Hydroxyamphetamine	0.010
10	DL-1-Amino-*p*-hydroxyphenylphosphate	0.017
11	L-Tyrosinol adenylate	0.000023
12	D-Tyrosinol adenylate	0.00032

[a]Limiting K_m value;[52] K_m value in standard assay is 9.0×10^{-6} *M* (lit.[39] K_m, 6.1×10^{-6} *M*). [b]Lit.[39] K_i, 6.0×10^{-6} *M*. [c]Lit.[39] K_i, 4.1×10^{-6} *M*. [d]Lit.[39] K_i, 1.7×10^{-5} *M*. [e]Lit.[39] K_i, 8.1×10^{-6} *M*. [f]Lit.[42] K_i, 2.9×10^{-8} *M*.

Table III. Inhibition[a] of ATP-PP_i Exchange by

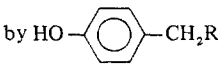


No.	R	K_i, m*M*
13	$-OCH_2CH_3$	0.10[b]
14	$-O(CH_2)_2CH_3$	0.09
15	$-O(CH_2)_3CH_3$	0.04
16	$-OCH(CH_3)CH_2CH_3$	0.09
17	$-OC(CH_3)_3$	0.2
18	$-OCH(CH_3)(CH_2)_2CH_3$	0.1
19	$-OCH_2C_6H_5$	0.03
20	$-NHCH_3$	0.22
21	$-NHCH_2CH_3$	0.17
22	$-NH(CH_2)_2CH_3$	0.23
23	$-NH(CH_2)_3CH_3$	0.43
24	$-NH(CH_2)_4CH_3$	1.4
25	$-NHCH_2C_6H_5$	0.063
26	$-NHCH_2(4\text{-}NO_2C_6H_4)$	0.065
27	$-NHCH_2(4\text{-}CH_3C_6H_4)$	0.078
28	$-NH(CH_2)_2C_6H_5$	0.038
29	$-NH(CH_2)_3C_6H_5$	0.18
30	$-NH(CH_2)_4C_6H_5$	0.36
31	$-NH(CH_2)_5C_6H_5$	0.34
32	$-NHC_6H_5$	0.20
33	$-NH(\beta\text{-}C_{10}H_7)$	0.096
34	Glycine	0.012
35	Alanine	0.005
36	Leucine	0.015
37	Valine	0.004
38	Isoleucine	0.008
39	Tyrosine	0.008
40	Glutamic acid	0.11
41	Glycylglycine	0.21

[a]All enantiomeric compounds listed are of the L configuration and exhibit competitive inhibition with tyrosine as the variable substrate. [b]Lit.[39] K_i, 2.0×10^{-5} *M*.

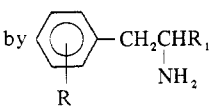
values only 4- to 13-fold larger than those of their respective L enantiomers.

Table III gives the inhibition properties of a series of esters and amides of L-tyrosine. Although the butyl (**15**) and benzyl (**19**) esters bind somewhat more effectively than the others, all of the esters bind at least tenfold poorer than the substrate. Although L-tyrosine amide (**7**) and hydroxamate (**8**) bind quite well, *N*-alkylamides show a reduced inhibitory effect which is similar for the methyl through *n*-propyl analogs. Further extension of the alkyl chain results in decreased affinity such that the *n*-pentylamide (**24**) binds 45-fold poorer than the parent tyrosine amide (**7**). The affinity of the benzyl (**25**) and phenylethyl (**28**) amides is greater than any of the *n*-alkylamides and approaches that of the unsubstituted amide (**7**). Substitution on the phenyl ring of **25** by either *p*-nitro (**26**) or *p*-methyl (**27**) groups has no effect on binding. The anilide (**32**) and β-methylamide (**33**) bind 56- and 27-fold poorer than substrate, respectively, and inhibit within the general range of potency observed for the phenylalkylamides.

Table IV. Inhibition[a] of ATP-PP_i Exchange by 

No.	R	Inhibition type	K_i, m*M*
42	$-CH_2COOH$	Noncompetitive	5.5
43	$-CH(OH)COOH$	Noncompetitive	8.1
44	$-COCOOH$	Noncompetitive	9.9
45	$-CH(NHCH_3)CH_3$	Competitive	7.3
46	$-CH[NHCH(CH_3)_2]CH_3$	Competitive	0.33
47	$-CH(NHCH_2C_6H_6)CH_3$	Competitive	2.1
48	$-N^+(CH_3)_3I^-$	Competitive	0.95
49	$-NH_2$	Competitive	0.13
50	$-CH_2CH_2NH_2$	Competitive	0.83

[a]Compounds are DL mixtures and, unless otherwise specified, exhibit competitive inhibition with respect to L-tyrosine.

Table V. Inhibition[a] of ATP-PP_i Exchange by 

No.	R	R_i	Enantiomer(s)	K_i, m*M*
51	H	$-COOH$	L	*b*
52	H	$-CH_2OH$	L	*b*
53	4-OCH_3	$-CH_3$	DL	*c*
54	4-OH, 3-OCH_3	$-CH_3$	DL	*c*
55	4-OH, 3-OH	$-H$	DL	2.5
56	4-OH, 2,3,5,6-F	$-COOH$	DL	10[d]

[a]Inhibition is competitive with respect to tyrosine. [b]No inhibition observed at 11 m*M*. [c]No inhibition observed at 5.0 m*M*. [d]Assayed at pH 5.3; K_m (L-tyrosine) = 0.34 m*M*.

With the exception of L-Tyr-Gly-Gly (**41**) and L-Tyr-L-Glu (**40**), the peptides of L-tyrosine examined bind much better than alkyl- or phenylalkylamides and approach or exceed the binding ability of L-tyrosine.

Inhibitory properties of a number of tyrosine analogs having a modified α-amino group are compared in Table IV; none of those listed supported ATP-PP_i exchange at 10 m*M* concentration in the standard assay. Substitution of the α-amino group by hydrogen (**42**), hydroxyl (**43**), or oxo (**44**) led to large losses in binding and, moreover, noncompetitive inhibition with respect to tyrosine. In contrast to the high affinity of peptides having tyrosine as the N-terminal amino acid, blocking the α-amino group by an amide linkage prevents binding.[39] Whereas tyramine (**2**) is a very good inhibitor, decreasing (**49**) or increasing (**50**) the chain length separating the amino and phenolic residues by one methylene group results in 23- and 148-fold losses, respectively, in binding. 4-Hydroxyamphetamine (**9**) binds about as well as tyrosine and provides a convenient analog to study the effect of N-alkylation on binding. Although the *N*-methyl derivative **45** binds 730-fold poorer than the unsubstituted compound **9**, some binding is regained upon substitution with more hydrophobic groups as in the *N*-benzyl (**47**) and *N*-isopropyl (**46**) analogs. The latter compound binds only 33-fold poorer than the parent 4-hydroxyamphetamine (**9**). Despite quaternization of the amine

Table VI. Binding of 6-Aminopurine Nucleotides and Nucleotide Analogs to TRS

No.	Compound	Inhibition type	K_i, m*M*
57	ATP	Substrate	0.50[a]
58	dATP	Competitive	3.0
59	α,β-CH_2-ATP	Competitive	12.2
60	ADP	Noncompetitive	6.4
61	AMP	Noncompetitive	6.3
62	3',5'-Cyclic AMP	Noncompetitive	33
63	Adenosine 5'-methylphosphonate	Noncompetitive	15
64	Adenosine 5'-ethylphosphonate	Noncompetitive	10
65	Adenosine 5'-propylphosphonate	Noncompetitive	11
66	Adenosine 5'-phenylphosphonate	Noncompetitive	20
67	Adenosine 5'-benzylphosphonate	Noncompetitive	8
68	Adenosine 5'-phenylethyl-phosphonate	Noncompetitive	8
69	Adenosine 5'-phenylpropyl-phosphonate	Noncompetitive	3
70	Adenosine 5'-phenylbutyl-phosphonate	Noncompetitive	3

[a] Lit.[39] K_m, 2.5×10^{-4} *M*.

Table VII. Noncompetitive Inhibition of ATP-PP_i Exchange by Adenosine and 9-Substituted Adenines

No.	Compound	K_i, m*M*
71	Adenosine	18
72	3'-Deoxyadenosine (cordycepin)	17
73	*cis*-4-(6-Amino-9*H*-purin-9-yl)-*trans*-2-*trans*-3-dihydroxycyclopentanemethanol	16
74	*cis*-9-(3-Hydroxymethylcyclopentyl)adenine	16
75	*cis*-9-(4-Hydroxymethylcyclohexyl)adenine	22
76	9-(3-Hydroxypropyl)adenine	68
77	9-(4-Hydroxybutyl)adenine	32
78	9-(5-Hydroxypentyl)adenine	47
79	9-(6-Hydroxyphexyl)adenine	27
80	Adenine	*a*
81	9-Isobutyladenine	40
82	9-Phenylbutyladenine	47

[a] No inhibition observed at 10 m*M*.

and the additional methyl groups, candicine iodide (**48**) binds eightfold better than the *N*-methyl analog **45**.

Table V contains inhibition data for a number of tyrosine analogs having a modified phenyl ring. Neither L-phenylalanine (**51**) nor L-phenylalanol (**52**) show inhibition at 11.0 m*M* in the assay and have K_i values estimated to be over 100 m*M*. Masking of the hydroxyl group of 4-hydroxyamphetamine (**9**) with a methyl ether (**53**) or placing a methoxy group (**54**) adjacent to it results in inactive compounds. Addition of a 3-hydroxyl group to tyramine (**2**) to give dopamine (**55**) results in a 2200-fold loss in binding relative to the unsubstituted compound. It is interesting to note that DOPA possesses substrate activity, albeit with a K_m value of 1.4 m*M*.[39] 2,3,5,6-Tetrafluorotyrosine (**56**) does not possess substrate or inhibitory properties at a concentration of 3.0 m*M* in the standard assay or at pH 6.0 (K_m for tyrosine at pH 6.0 is 7.6×10^{-5} *M*). However, at pH 5.3 where the phenolic group is *ca.* 61% ionized (pK_a = 5.1 for phenolic hydroxyl of **56**)[40] the compound (4.1 m*M*) shows competitive inhibition with respect to tyrosine with a K_i value of 1×10^{-2} *M* (K_m for L-tyrosine at pH 5.3 is 3.4×10^{-4} *M*).

Tables VI and VII give the inhibitory properties of a number of analogs of ATP. With the exception of dATP (**58**) and α,β-CH_2-ATP (**59**), competitive inhibitors relative to ATP, all the compounds in Table VI exhibit noncompetitive inhibition and possess K_i values greater than the K_m for ATP. The adenosine 5'-alkylphosphonates **63-65** have similar K_i values and appear to inhibit the enzyme slightly better than the phenylphosphonate (**66**) but poorer than the remaining phenylalkylphosphonates **67-70**. The latter compounds become slightly better inhibitors as the chain length is increased. Adenine (**80**) does not inhibit ATP-PP_i exchange at its upper limit of solubility (3 m*M*) in the assay mixture. Adenosine (**71**) and related cyclic and acyclic analogs (Table VI) bind poorly and are noncompetitive inhibitors with respect to ATP.

Discussion

Optimal interactions within an enzyme-substrate complex require that regions of the enzyme complement the binding points of the substrate in topography, polarity, charge, and solvation. Often, the construction of the active site may not be optimal for binding of the substrate but includes structural features which have evolved in such a way to provide maximal specificity and catalytic efficiency. Analysis of the binding properties of structural analogs of a substrate provides an approach to probing these features in a manner which is not amenable to other physical measurements. In addition, a study of structural analogs often uncovers information about regions surrounding the active site which may be useful in the design of potent and specific enzyme inhibitors and in comparative studies which seek to uncover subtle differences among related enzymes. A complete discussion of problems inherent in this approach with specific reference to the aa-tRNA synthetases may be found in earlier reports.[3,41]

One of the general properties of substrate interactions with the synthetases which is being uncovered is that the carboxylate group of the substrate amino acid is not an essential binding point. Numerous analogs in which the carboxylate group has been omitted or modified have been shown to be potent inhibitors of these enzymes.[4,5,42-46] It does appear that various synthetases, even substrate identical enzymes from different sources,[45,46] may respond differently toward such analogs. From these studies, it appears that the carboxylate binding regions of these enzymes may differ in environment and function. From observations that hydrophobic substituents bind to PRS from *E. coli* much more effectively than the carboxylate of phenylalanine,[3] it has been suggested that the enzyme active site serves to partially desolvate the carboxylate of the substrate upon binding. In this manner, catalytic efficiency could be enhanced at the expense of binding energy. In the work described here, it is shown that omission of the carboxylate of tyrosine provides an inhibitor which binds as effectively as the substrate. In further contrast to the hydrophobic nature of the carboxylate binding region of the phenylalanine activating enzyme, substitution of the carboxylate of L-tyrosine by groups of widely disparate polarity has little effect on binding. Thus, in addition to the fact that the carboxylate of tyrosine is not necessary for binding, it probably occupies a noncontact area of the enzyme which, in probable contrast to PRS, causes no perturbation of solvation upon complexation.

The series of esters and amides given in Table III was evaluated to probe regions of the enzyme adjacent to the site occupied by the carboxylate group of tyrosine. A detrimental effect on binding, amounting to about 1 kcal/mol, is obtained with the first methylene group extending from the noncontact area, as observed with the methylamide (**20**) and methyl ester (**5**) of tyrosine. Further extension of

an alkyl chain up to five carbon atoms has little additional effect upon binding. The recovery of *ca.* 1 kcal/mol in binding energy which is observed with the benzylamide (**25**) or ester (**19**), and the phenylethylamide (**28**) of tyrosine is probably the result of a specific interaction between the phenyl moieties and a restricted hydrophobic region of the enzyme, since the effect is not observed with phenylalkylamides of longer chain length. Interestingly, with the exception of L-Tyr-Gly-Gly (**41**) and L-Tyr-L-Glu (**40**), the affinity of dipeptides having tyrosine as the N-terminal amino acid is much greater than the alkyl- or phenylalkylamides and may approach or exceed that of the substrate. Since the nature of the aliphatic chain on tyrosine amides does not appear to be a major determinant for binding, the α-carboxylate of the carboxyl terminal amino acid must be responsible for the nominal 2 kcal/mol increase in binding energy which is observed with these dipeptides. It is tempting to speculate that the carboxylate moiety of these inhibitors interacts with a region of the enzyme which normally binds to one of the phosphate groups of ATP.

Comparison of binding properties of amides of L-tyrosine and L-phenylalanine to their cognate synthetases indicates that the regions extending from the carboxylate sites of these two enzymes are also considerably different. With the phenylalanine activating enzyme, attachment of an sp^3-hybridized carbon to the amide nitrogen of L-phenylalanine amide results in a much greater loss in binding,[3] indicating that this region is more sterically restricted than the analogous region of TRS. In further contrast, the affinities of *N*-alkyl- and *N*-phenylalkylamides for PRS systematically increase with increasing chain length, and phenylalanine peptides are only poorly bound to PRS.

The protonated α-amino group of amino acids appears to be a major binding locus which is essential for substrate activity for a number of aa-tRNA synthetases.[3-5] Calendar and Berg[39] have reported that gross modification of the α-amino group of tyrosine, *i.e.*, replacement by hydrogen, hydroxyl, oxo, or acylamido, results in compounds which are inactive both as substrates and inhibitors for TRS of *E. coli* and *Bacillus subtilis*. It is shown here that high concentrations of these compounds result in weak, noncompetitive inhibition, suggesting that they do not interact with the substrate binding site. Inhibition by candicine iodide (**48**) provides strong experimental support that tyrosine analogs are bound as their protonated forms and suggests the occurrence of an ionic interaction with a negatively charged site on the enzyme. Substitution of *N*-alkyl groups on *p*-hydroxyamphetamine (**9**) results in a general loss of binding, although much of the decrease appears to be recoverable by increasing the nonpolar nature of the substituent. It is noted that the additional binding power of the *N*-isopropyl analog **46** over the *N*-methyl analog **45** corresponds to *ca.* 1 kcal/mol/methyl, approximately what is expected from optimal hydrophobic interactions.[47]

As with other aa-tRNA synthetases,[3,5,48,49] the optimal separation between the side chain and amino binding points of tyrosine analogs is that which is found in the natural substrate. Lengthening or shortening the distance separating these binding points in tyramine by one methylene group results in 148- and 23-fold decreases in binding, respectively. Parallel results have been obtained with the valine activating enzyme[5] but the higher and lower homologs of 2-phenethylamine bind identically to PRS.[3]

In agreement with the conclusions of Calendar and Berg,[39] the phenol group, as found in the natural substrate, is necessary for substrate activity. Of the phenyl-substituted analogs examined (Table V), only dopamine (**55**) demonstrated significant inhibition at pH 7, binding about as well as the false-substrate DOPA. Since the fidelity of protein synthesis requires exact recognition of each of the amino acids by its cognate synthetase, it is not surprising that the *E. coli* TRS is not inhibited by high concentrations of phenylalanine or phenylalanol. From the data given, it may be calculated that binding of these analogs is at least 10^4 times poorer than their tyrosine counterparts. The nominal 6 kcal/mol in binding energy which must be attributed to the phenolic hydroxyl group of tyrosine is well suited for purposes of biological specificity but difficult to explain in terms of current concepts of intermolecular interactions in water.[47] Utilizing upper estimates for the magnitude of hydrogen bonding, hydrophobic forces, and polarizability, we estimate that the phenolic hydroxyl group should not contribute more than 2.15 kcal/mol in binding energy to TRS. It must be concluded that factors other than specific interactions of the phenolic hydroxyl of tyrosine contribute to specificity and play an important role in discriminating between phenylalanine and tyrosine.

Tetrafluorotyrosine (**56**) is neither substrate nor inhibitor at pH values where the phenol is ionized. However, at pH values where a significant amount of the neutral phenol is present, the fluorinated analog is a competitive inhibitor with respect to tyrosine. These results indicate that the phenolic hydroxyl of tyrosine only binds to TRS as the neutral form and does not act as a hydrogen bond acceptor; if the latter were true, the anionic form of **56** might be expected to bind more tightly than the neutral species.

The aa-tRNA synthetases generally demonstrate stringent stereochemical specificities for their natural L-amino acid substrates which usually extends to the binding power of analogs.[3-5] It is shown here that analogs of D-tyrosine in which the carboxylate has been replaced by other substituents bind only slightly poorer than the corresponding L enantiomers. This is not initially surprising in view of the proposal that the carboxylate group of tyrosine is in a noncontact area when bound to TRS; in the D enantiomers of the analogs examined, the α substituent could project into the region which is normally occupied by the α hydrogen of the natural substrate. However, this possibility does not seem tenable with the observation that TRS catalyzes both the activation and transfer to $tRNA^{Tyr}$ of D-tyrosine;[39,50] it would appear reasonable that the carboxylate of both enantiomers would have to occupy the same catalytic site when bound to the enzyme in order to show substrate activity. The question then arises as to how the D and L enantiomers could have the amino and phenolic groups rigidly bound and still project the carboxylate group into a common catalytic region. In an attempt to resolve this problem, we sought to introduce an additional binding point which would establish a three-point contact and impose rigid restraints on the conformational mobility when bound to the enzyme. Esterification of L-tyrosinol with AMP results in a 700-fold increase in binding and indicates strong binding loci at the phenol, α-amino, and AMP moieties; the close structural resemblance of this analog to the tyrosyladenylate intermediate leaves little doubt that it binds in a fashion analogous to the substrates. The binding energy of D-tyrosinol adenylate is only *ca.* 1.5 kcal/mol less than the L isomer and roughly parallels the differences observed among the D- and L-tyrosinols (0.9 kcal/mol), tyrosines (1.9 kcal/mol), and tyrosine methyl esters (1.0 kcal/mol). It should

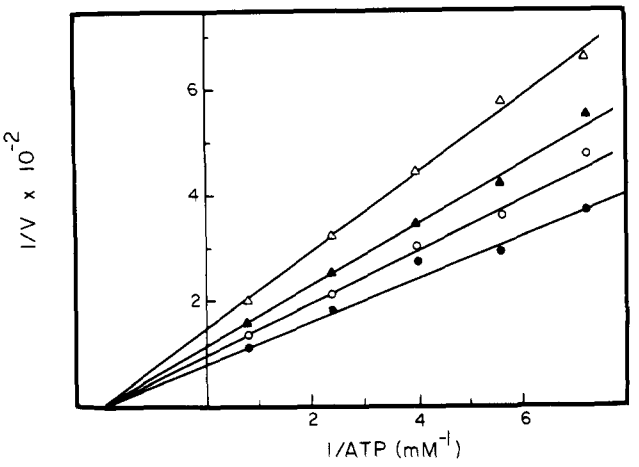


Figure 1. Inhibition of ATP-PP_i exchange by adenosine, varying ATP concentration in the absence of inhibitor (•), and at 6.0 (○), 9.0 (▲), and 12.0 m*M* (△) of adenosine.

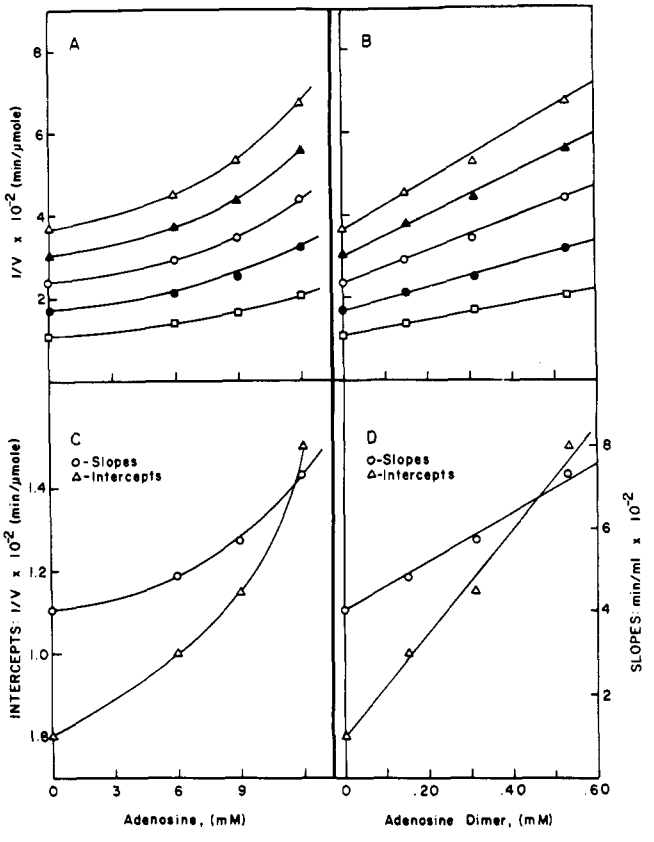


Figure 2. (A,B) Plots of reciprocal velocity of ATP-PP_i exchange *vs.* concentration of adenosine monomer and dimer at 1.25 (□), 0.42 (•), 0.25 (○), 0.18 (▲), and 0.14 m*M* (△) of ATP. (C,D) Slope-intercept replots of data given in A and B.

be noted that the three-point contact must still occur with D-tyrosinol adenylate since the analog binds significantly better (50-fold) than the potent inhibitor, L-tyrosinol (**3**). Molecular models of the enantiomeric tyrosinol adenylates illustrate that if the three-contact points (*viz.* amino, phenol, and adenylate) are rigidly bound to the enzyme as the strong binding indicates, the α-CH_2 groups could occupy the same region if a small degree of rotation were permitted about the C_α-C_β bond of the tyrosinol moiety; a similar effect could be accomplished by an analogous conformational change of the enzyme. On the basis of the above argument, it may be proposed that the carboxylate of D-tyrosine may occupy the same site as the carboxylate of L-tyrosine and thus behave as a substrate, but only at the expense of binding energy necessary to (a) rotate the C_α-$_\beta$ bond in a manner conducive to amino and phenol binding, or (b) cause a conformational change of the enzyme in order to juxtapose catalytic groups in their proper positions.

With the exception of dATP (**58**) and α,β-CH_2-ATP (**59**), none of the ATP analogs examined were competitive inhibitors with respect to ATP. The ATP binding site of TRS appears to require the intact triphosphate moiety, probably as its magnesium complex. Similar properties have been observed with the valine and isoleucine activating enzymes,§ but with PRS, the phosphate groups of ATP are detrimental to binding and nucleoside analogs are potent inhibitors.[3] It is interesting to note that those enzymes which have been shown to require the triphosphate groups of ATP for binding are inhibited by PP_i.[51,52] In contrast, ATP binding to PRS is not inhibited by PP_i.[41] This feature may be of diagnostic utility in classifying the aa-tRNA synthetase ATP binding sites. All other nucleotide analogs examined show weak noncompetitive inhibition of TRS. Although binding of the adenosine 5′-phosphonates does increase with longer chain lengths, little can be discerned concerning the nature of complexation of these compounds.

In order to obtain more information about the interactions of ATP analogs not possessing the triphosphate moiety, the kinetics of inhibition by adenosine (Figure 1) were examined in more detail. A plot of reciprocal velocity *vs.* adenosine concentration (Figure 2A) as well as slope and intercept replots of the primary data (Figure 2C) gave curved lines and suggests the possibility that more than one molecule of adenosine combines with the enzyme.[53] It has been established that adenosine forms stacks in aqueous solution at the concentrations employed in these studies.[54,55] Indeed, when similar replots were constructed using the calculated amount of adenosine dimer present in solution, a family of straight lines was obtained (Figure 2B,D). From these data, it is tempting to suggest that these analogs bind as dimers at a locus remote from the ATP binding site, perhaps in a region normally occupied by $tRNA^{Tyr}$. This possibility is currently under investigation.

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Notes

Synthesis of a Proposed Pepsitensin

Antonio C. M. Paiva,* Therezinha B. Paiva, and Maria E. Miyamoto

Department of Biophysics and Physiology, Escola Paulista de Medicina, 04023 São Paulo, Brazil.
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Pepsitensin is a vasopressor peptide obtained by peptic proteolysis of the α_2-globulin fraction of blood plasma.[1] Its biological activities are qualitatively identical with those of angiotensin II[2] but the two peptides were shown to be different.[3] Franze, *et al.*,[4] isolated from plasma, after incubation with pepsin at pH 6, a pressor substance that was identified as the decapeptide angiotensin I. More recently, a pepsitensin was isolated from bovine plasma after hydrolysis with pepsin at pH 3, for which the following sequence was proposed: Asp·Arg·Val·Tyr·Val·His·Pro·Phe·His·Leu·Leu (**1**).[5] These results might indicate that peptic proteolysis of the plasma protein substrate would differ according to whether pH 6 or 3 is employed in the incubation, leading to the production of either angiotensin I or the undecapeptide,[5] respectively.

In order to verify this hypothesis, and to study the pharmacological properties of the proposed pepsitensin, we have synthesized both **1** and its Ile[5] analog **2**, which would be the homologous peptide obtained from horse plasma.

The peptides were tested for their biological activity on the rat's blood pressure, the isolated guinea pig ileum, and the isolated rat uterus using a four-point assay design,[6] with the results shown on Table I. The activities of the two peptides on the rat's blood pressure and the guinea pig ileum were surprisingly low compared to angiotensin I,[7] from which they differ only by an additional leucine residue at the C terminus. The observed activities appear to be intrinsic to **1** and **2**, and not due to their conversion to angiotensin II, because of the great differences in "converting enzyme" activity in the rat's blood plasma, uterus, and the guinea pig ileum.[8]

The conversion of both **1** and **2** to the respective angiotensin II octapeptides was easily obtained by the action of carboxypeptidase. An illustration of this conversion is presented in Figure 1 which shows that the incubation of **1** with porcine carboxypeptidase A resulted in a very large

Table I. Biological Activity[a] of Proposed Pepsintensins

Assay	Compd **1**[b]	Compd **2**[b]
Rat's blood pressure	1.28 (0.08)	1.42 (0.09)
Rat uterus	0.82 (0.10)	1.40 (0.06)
Guinea pig ileum	0.88 (0.09)	1.68 (0.06)

[a] Relative to Ile[5]-angiotensin II = 100, on a molar basis. [b] Each value was obtained from a four-point assay made with at least eight groups of four doses. Standard deviations are shown inside parentheses.