Phenylalanyl Transfer Ribonucleic Acid Synthetase from *Escherichia coli*. Analysis of the Phenylalanine Binding Site*

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ABSTRACT: Using structural analogs of L-phenylalanine, a model has been constructed which describes the amino acid binding site of Phe-tRNA synthetase from *Escherichia coli* B. The area of the enzyme which complexes the benzene ring of L-phenylalanine represents the primary site of recognition and one of the two major binding loci. This region is best described as a hydrophobic pocket with a stringent steric requirement for the phenyl ring of the substrate; substituents on the benzene ring which are larger than hydrogen invariably lead to a loss of substrate activity and binding energy. The other major binding locus is that which complexes the protonated amino group of L-phenylalanine and related analogs, and is probably best represented as an anionic group of the enzyme. This region also has rigid steric requirements for binding and substrate activity and is intolerant of substituents on the amine which are larger than hydrogen. The stereospecificity of the enzyme is exact with regard to substrate and binding properties and appears to be governed by steric constraints in the region of the binding site which is occupied by the α hydrogen of L-phenylalanine; groups larger than hydrogen perturb other binding loci and lead to large losses in affinity. The presence of the α-carboxyl group is not necessary for optimal binding, and substitution by hydrophobic groups leads to substantial increases in affinity for the enzyme. This has been interpreted as evidence that this region of the enzyme is partially hydrophobic, a property which may be common to many activating enzymes and which may play an important role in the catalytic reaction. Although L-phenylalaninamide is a good inhibitor of ATP–PP_i exchange, attachment of an sp^3-hybridized carbon to the amide nitrogen results in 857-fold increase in K_i because of a very unfavorable steric interaction. Evidence is also presented for a diffuse hydrophobic region adjacent to the site which proximates the carboxylate of L-phenylalanine which may be utilized to compensate for the detrimental steric interaction. The effects of conformational changes, distortion of inhibitors, and secondary perturbations of binding loci upon interpretations of intermolecular forces are discussed.

The correct translation of genetic information is contingent upon the exactness with which each of the aa-tRNA[1] synthetases attaches an amino acid to the tRNAs which translate the codons for that amino acid. Should a mistake occur at this stage, the amino acid would inevitably be incorporated into an incorrect position in a protein (Chapeville *et al.*, 1962). A pertinent aspect regarding the specificity of protein biosynthesis resides in the mechanism by which these enzymes recognize the correct amino acids and tRNA molecules. The stringent specificity for a particular amino acid may be envisaged as manifestations of three safeguards inherent in each of the synthetases: the amino acid must be recognized and reversibly bound to the enzyme, activated to form an aminoacyladenylate, and transferred to bound tRNA. With regard to naturally occurring amino acids, each stage in this sequence appears to be more specific than the previous, and in concert they serve as an effective filter for all but the correct aa-tRNA.

A number of amino acid analogs are able to meet the requirements for the binding and activation steps, and in some instances are esterified to tRNA (see, for example, Owens and Bell, 1970, Calendar and Berg, 1966, Conway *et al.*, 1962, Papas and Mehler, 1970, Mitra and Mehler, 1967, and Stern and Mehler, 1965); for most of these, specificity is still maintained to some degree, manifested as decreased maximal velocity or increased K_m. With a few exceptions (Norris and Berg, 1964; Bergmann *et al.*, 1961), the naturally occurring amino acids are activated only by their corresponding synthetases, and the final safeguard provided for in the transfer step is rarely utilized *in vivo*. Furthermore, since a given synthetase is not inhibited by the nineteen nonsubstrate amino acids, it is probably true that the major factor for *in vivo* specificity resides in binding of the amino acid rather than the activation step. Clearly, an understanding of the molecular basis for specificity of these enzymes requires a knowledge of the intermolecular forces which lead to substrate binding, and the differences in the binding sites among the various ligases. In the present work, competitive inhibitors are used to analyze the topography and localized environment of the L-phenylalanine binding site of PRS[1] from *E. coli* B. Comparisons of the results obtained with similar studies of other activating enzymes (Papas and Mehler, 1970; Owens and Bell, 1970; Calendar and Berg, 1966) permit speculation on features which may be relevant to general questions of catalysis and specificity.

Materials and Methods

Phe-tRNA synthetase was isolated from *E. coli* B (General Biochemicals) by the method of Stulberg (1967) and ATP–[^{32}P]PP_i exchange assays were performed under the standard conditions described in the accompanying paper (Santi *et al.*, 1971). Inhibition constants (K_i) were obtained by double-reciprocal plots (Lineweaver and Burk, 1934) varying L-phenyl-

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[1] Abbreviations used are: PRS, Phe-tRNA synthetase (EC 6.1.1.4) from *Escherichia coli* B; 3,5-DNBA, 3,5-dinitrobenzoic acid; aa-tRNA, aminoacyl-tRNA.

TABLE I: Substituted D,L-Phenylalaninols, $RC_6H_4CH_2CH(NH_2)CH_2OH$, Prepared by Reduction of Corresponding Amino Acid.[a]

	R	Mp (°C)	Yield (%)	Recrystallization Solvent
XXX	*o*-F	83–84.5	60	Benzene
XXXI	*m*-F	74.5–76.5	62	Benzene
XXXII	*p*-F	92.5–94.5	50	Benzene
XXIV	*o*-Cl	118–119.5	79	Benzene
XXV	*m*-Cl	185–187 (oxalate)	60	EtOH
		217–219 (hydrochloride)	40	EtOH
XXVI	*p*-Cl	80–83	78	Benzene
XXVII	*o*-CH_3	205–207 (hydrochloride)	45	*i*-PrOH
XXVIII	*m*-CH_3	222–223 (hydrochloride)	62	*i*-PrOH
XXIX	*p*-CH_3	224–227 (hydrochloride)	35	*i*-PrOH
		174–176 (oxalate)	60	EtOH

[a] Elemental analyses were within 0.3% of theoretical.

TABLE II: Properties of Alkyl- and Phenylalkylamides, $C_6H_5CH_2CH(NH_2)C(=O)NHR$, of L-Phenylalanine.[a]

	R	Salt	Mp (°C)	Yield (%)[b]	Recrystallization Solvent
LX	CH_3	HCl	204–206	54	*i*-PrOH–Et_2O
LXI	CH_2CH_3	HCl	187–188	21	*n*-BuOH–Et_2O
LXII	$(CH_2)_2CH_3$	HCl	155–157	44	*i*-PrOH–Et_2O
LXIII	$(CH_2)_3CH_3$	HCl	146–147	47	*n*-BuOH–Et_2O
LXIV	$(CH_2)_4CH_3$	HCl	137–138	38	*i*-PrOH–Et_2O
LXVI	$CH_2C_6H_5$	HCl	177–179	39	*i*-PrOH
LXVII	$(CH_2)_2C_6H_5$	*c*	70–71[d]	34	Cyclohexane
LXVIII	$(CH_2)_3C_6H_5$	HCl	135–136	39	*i*-PrOH–Et_2O
LXIX	$(CH_2)_4C_6H_5$	HBr	137–139[d]	37	*i*-PrOH
LXX	$(CH_2)_5C_6H_5$	3,5-DNBA	142–143	76	*i*-PrOH

[a] Elemental analysis were within 0.3% of theoretical. [b] Tlc (silica gel) showed one spot on $CHCl_3$–EtOH (9:1, v/v). [c] Isolated as free base. [d] Solvent system used for tlc was $CHCl_3$–EtOH (19:1, v/v).

alanine at fixed levels of ATP (4.0 mM), PP_i (2.0 mM), and magnesium chloride (21 mM). $I/S_{0.5}$ values refer to the inhibitor: phenylalanine ratio necessary to produce 50% inhibition in the standard ATP–PP_i exchange assay. Unless otherwise specified, where K_i values are given the inhibitors were competitive with respect to phenylalanine.

ATP, L-phenylalanine, L-alanine, *p*-chlorophenylalanine, *o*-, *m*-, and *p*-fluorophenylalanine, L-phenylalaninamide, D-3-phenyl-2-aminopropane (D-amphetamine), phenyllactic acid, phenylpyruvic acid, tyramine, L-tyrosine, and L-phenylalanine dipeptides were products of Sigma Chemical Co. L-Tyrosine was separated from an impurity which stimulated ATP–PP_i exchange by repetitive development on Whatman No. 3MM sheets using 1-butanol–acetic acid–water (9:1:1, v/v). 2-Phenylethylamine, L-3-phenyl-2-aminopropane (L-amphetamine), 1,2-diphenylethylamine, α,α,N-trimethylphenylethylamine, *N*-methyl-2-phenylethylamine, benzylamine, 3-phenylpropylamine (purified as their hydrochloride salts), and hydrocinnamic acid were obtained from Aldrich Chemical Co. 3,4-Dihydroxyphenylethylamine (dopamine) and L-tyrosinol were purchased from Nutritional Biochemicals Corp., and D-phenylalanine was obtained from Mann Research Laboratories. D,L-1-Trifluoromethyl-2-phenylethylamine and all amphetamine analogs listed in Table V were gifts from Smith, Kline and French Co. (*R*)(+)- and (*R,S*)(±)-α-methylphenylalaninol and α-methyl-L-phenylalanine were generously supplied by S. Terashima and S. Yamada. Substituted phenylalanines not available commercially were prepared by the method of Albertson and Archer (1945). *N*-Alkylphenylalanines were prepared by the method of Quitt *et al.* (1963). D- and L-phenylalaninol (mp 91–92°) were prepared by $LiAlH_4$ reduction of tristrimethylsilyl-D-and -L-phenylalanine as described by Venkateswaran and Bardos (1967). The 2-amino-3-arylpropanols listed in Table I were prepared by the same procedure. In cases where the free bases could not readily be crystallized, the products were isolated as oxalate salts by treatment with a saturated solution of oxalic acid in ethanol, or as hydrochloride salts by passing hydrogen chloride gas through an ethereal solution of the free base. Early in this work oxalate was found to inhibit ATP–PP_i exchange and all oxalate salts were converted to hydrochlorides prior to assay as follows. A solution of the oxalate salt (2 mmoles) in 15 ml of 1 N sodium hydroxide was extracted with three 20-ml portions of ether. The organic layer was dried over sodium sulfate, filtered, and saturated with hydrogen chloride. The precipitate was collected and recrystallized to constant melting point.

The N-substituted L-phenylalaninamides listed in Table II were prepared by the following general procedure.

L-*Phenylalanine Anilide* (*LXV*). To a stirred solution of 820 mg (2.7 mmoles) of carbobenzoxy-L-phenylalanine and 0.373 ml (2.7 mmoles) of triethylamine in 20 ml of dry tetrahydrofuran at −5° was added 368 mg (2.7 mmoles) of isobutyl chloroformate. After 20 min, 250 mg (2.7 mmoles) of aniline was added and stirring continued for 1 hr at −5°. After an additional 2 hr at ambient temperature, 50 ml of water was added and most of the tetrahydrofuran was removed by spin evaporation. The precipitated product was filtered and dissolved in 50 ml of chloroform. After successive washings with 50-ml portions of 0.1 N HCl, 5% K_2CO_3, and water, the solution was dried ($MgSO_4$) and evaporated at reduced pressure. The residue was recrystallized twice from benzene to give 700 mg (70%) of white crystals, mp 166–168°; tlc using chloroform–ethanol (9:1, v/v) showed one spot ($R_F \simeq 0.8$). A solution of 500 mg (1.33 mmoles) of Cbz-phenylalanineanilide in 100 ml of methanol and 2 ml of glacial acetic acid was hydrogenated over 0.1 g of 10% Pd/charcoal for 12 hr. After removal of the catalyst, the solvent was evaporated *in vacuo*, and the residue was dissolved in chloroform and washed with

TABLE III: Stimulation and Inhibition of ATP–PP_i Exchange by Substituted Phenylalanines, $RC_6H_4CH_2CH(NH_2)CO_2H$.

	R[a]	Activation[b]	% Inhibn at 12 mM
I	H	+ (K_m = 0.05 mM)	
II	*o*-Cl	−	0
III	*m*-Cl	−	34
IV	*p*-Cl	−	29 (K_i = 6.8 mM)
V	*o*-NO_2	−	0
VI	*m*-NO_2	−	15
VII	*p*-NO_2	−	0
VIII	*o*-CH_3	−	5
IX	*m*-CH_3	−	11
X	*p*-CH_3	−	0
XI	*o*-NH_2	−	59 (K_i = 2.4 mM)
XII	*m*-NH_2	−	10
XIII	*p*-NH_2	−	5
XIV	*o*-F	+ (K_m = 5.0 mM)[c]	
XV	*m*-F	+ (K_m = 1.4 mM)[c]	
XVI	*p*-F	+ (K_m = 0.56 mM)[c]	
XVII	*p*-OH	−	

[a] With the exception of L-phenylalanine (I), all compounds were D,L mixtures. [b] Reactions were run under standard assay conditions substituting the analog for L-phenylalanine at 1.0 and 12.0 mM. [c] Identical V_{max} values were obtained for XIV, XV, and XVI; K_m values are for D,L mixture and should be divided by two to obtain the value for the substrate L enantiomer.

5% $KHCO_3$ and water. The dried ($MgSO_4$) solution was evaporated *in vacuo*, and the residue twice recrystallized from ethanol–water to give 220 mg (69%) of L-phenylalanine anilide, mp 69–70° (lit. mp 72° (Schallenberg and Calvin, 1955)).

Results

Substrate and inhibitory properties exhibited by a series of ortho-, meta-, and para-substituted phenylalanines in the PRS catalyzed ATP–PP_i exchange are given in Table III. With the exception of fluorine, substituents on the phenyl ring prevented the exchange reaction from occurring. The *o*-, *m*-, and *p*-fluorophenylalanines exhibited varying K_m values but had identical V_{max}, suggesting that the effect of the fluorine substituent is on the affinity of the analog for PRS. The poor inhibitory properties and limited water solubility of most of the β-arylalanines examined prohibited the determination of accurate K_i values or the type of inhibition. In the two instances where K_i values could be obtained (IV and XI), the inhibition was shown to be competitive with respect to L-phenylalanine. A hydroxyl group in the para position to give L-tyrosine (XVI) completely eliminates binding even at high concentrations. Not listed in Table III is L-alanine, which at 12 mM caused neither stimulation nor inhibition of ATP–PP_i exchange.

Since one of the objectives of this investigation was to quantitate the effects of phenyl substituents upon binding, it was necessary to enhance the affinity of these analogs for PRS by optimizing interactions at some other part of the molecule. A number of observations (see Discussion) suggested that the carboxyl moiety of phenylalanine could be modified to provide potent inhibitors of PRS which bind to the same site as the substrate amino acid. In Table IV, inhibition constants and negative free energies of binding ($-\Delta F$) are given for a number of phenylalanine analogs in which the carboxylate group is replaced by other functions. As expected of the random order of substrate addition (Santi *et al.*, 1971) all the analogs listed were shown to be competitive inhibitors with respect to phenylalanine and noncompetitive with respect to ATP.

TABLE IV: Inhibition of ATP–PP_i Exchange by $C_6H_5CH_2C(NH_2)HR$.

	R	K_i[a] (mM)	$-\Delta F$ (kcal/mole)
XVIII	$CONH_2$	0.035	6.30
XIX	CH_2OH	0.006	7.38
XX	CH_3	0.012	6.95
XXI	H	0.093	5.70
XXII	C_6H_5	0.14[b]	5.47
XXIII	CF_3	1.0[b]	4.25

[a] Values are given for L enantiomer. [b] Calculated from D,L mixture.

Inhibition constants for a number of ortho-, meta- and para-substituted D,L-phenylalaninols and D,L-amphetamines are given in Table V. Comparison with Table II shows that, in all cases examined, substitution of hydroxymethyl or methyl for the carboxylate group of corresponding phenylalanine analogs results in considerable enhancement of binding to PRS. Tolerance for bulkier substituents is slightly greater at the meta position, and binding in relation to the substituents used follows the general order of H > F > Cl > CH_3,CF_3. Tyrosinol

TABLE V: Inhibitory Power of Phenyl-Substituted Derivatives of D,L-Phenylalaninol and D,L-Amphetamine.

$RC_6H_4CH_2CH(NH_2)CH_2OH$ and $RC_6H_4CH_2CH(NH_2)CH_3$		R	K_i (mM)
A. D,L-Phenylalaninols			
	XXIV	*o*-Cl	1.3
	XXV	*m*-Cl	0.70
	XXVI	*p*-Cl	2.8
	XXVII	*o*-CH_3	8.2
	XXVIII	*m*-CH_3	4.0
	XXIX	*p*-CH_3	9.7
	XXX	*o*-F	0.41
	XXXI	*m*-F	0.37
	XXXII	*p*-F	0.21
	XXXIII	*p*-OH	
B. D,L-Amphetamines			
	XXXIV	*p*-Cl	3.8
	XXXV	*o*-CH_3	9.4
	XXXVI	*p*-CH_3	4.7
	XXXVII	*o*-CF_3	10
	XXXVIII	*p*-CF_3	12
	XXXIX	*p*-F	0.48
	XL	*p*-OH	Noncompetitive

TABLE VI: Stereospecificity of PRS; Inhibition of ATP–PP_i Exchange by $C_6H_5CH_2C(NH_3^+)R_1R_2$.

	Compound	R_1	R_2	K_i (mM)	$-\Delta F$ (kcal/mole)
XXI	2-Phenylethylamine	H	H	0.093	5.72
XLI	D-Phenylalanine	H	CO_2H	8.5[a]	2.94
XLII	D,L-Phenylalaninol	CH_2OH(H)	H(CH_2OH)	0.01	7.09
XXI	L-Phenylalaninol	CH_2OH	H	0.006	7.41
XLII	D-Phenylalaninol	H	CH_2OH	1.42	4.04
XX	L-Amphetamine	CH_3	H	0.01	7.09
XLIII	D-Amphetamine	H	CH_3	0.19	5.28
XLIV	α,α-Dimethylphenylethylamine	CH_3	CH_3	0.48	4.71
XLV	(*R*)(+)-α-Methylphenylalaninol	CH_3	CH_2OH	0.74	4.40
XLVI	(*S*)(−)-α-Methylphenylalaninol	CH_2OH	CH_3	0.37[b]	4.87
XLVII	(*R,S*)(±)-α-Methylphenylalaninol	CH_3(CH_2OH)	CH_2OH(CH_3)	0.49	4.69
XLVIII	(*R*)(+)-α-Methylphenylalanine	CO_2H	CH_3	2.0[a]	3.83

[a] Estimated from per cent inhibition. [b] K_i value calculated using the expression $K^{DL} = 2K^LK^D/(L^L + K^D)$ (H. J. Schaeffer and M. A. Schwartz, private communication) and measured values for XLV and XLVII.

(XXXIII) and *p*-hydroxyamphetamine (XL) are poorly bound to PRS. Furthermore, XL and tyramine (not listed) were shown to be noncompetitive inhibitors with respect to L-phenylalanine.

Listed in Table VI are K_i values and calculated free energies of binding which result from substitution of various groups for one or both of the α hydrogens of 2-phenylethylamine (XXI). D-Phenylalanine (XLI) did not stimulate ATP–PP_i exchange at concentrations as high as 12 mM and is a poor inhibitor of PRS. Stereoselectivity is also retained with the phenylalanine analogs examined; substitution of the α hydrogen of L-phenylalanine or corresponding L analogs by larger groups invariably leads to large losses in binding. L-Phenylalaninol (XXI), where hydroxymethyl replaces the carboxylate of the substrate, binds twice as well as the D,L mixture (XLII) and some 230-fold more tightly than the D enantiomer (XLII). Although the difference is not as pronounced, substitution by methyl groups at the α position to give XX and XLIII has a similar effect.

In order to evaluate the nature and importance of the interaction of the α-amino group of phenylalanine with PRS in the enzyme–substrate complex, a number of phenylalanine analogs in which the α-amino group had been replaced by other substituents were examined (Table VII). None of the analogs studied stimulated ATP–PP_i exchange at concentrations up to 12 mM. Replacement of the amino group by hydrogen (XLIX), hydroxyl (L), or oxo (LI) causes large losses in binding, giving K_i values 45- to 200-fold greater than K_m for L-phenylalanine. Shortening or lengthening the carbon chain separating the α-amino and phenyl moieties by a methylene group results in approximately 20-fold losses in binding (compare LI and LIII to XXI). The 360-fold difference between K_i of *N*-methylphenylalanine (LIV) and K_m of phenylalanine, as well as the lack of any inhibition by LV and LVI at $I/S = 1.7$, demonstrates that placement of alkyl substituents on the amino group results in severe losses in binding. As expected from structure–binding relationships, the *N*-alkyl derivatives of amphetamine (LVIII, LIX) and 2-phenylethylamine (LVII) bind better than the corresponding *N*-alkylphenylalanines but much poorer than the parent primary amines.

As previously shown (Table III), L-phenylalaninamide (XVIII) is a good inhibitor of PRS-catalyzed ATP–PP_i exchange. In order to examine the region of PRS which is adjacent to the carboxylate of L-phenylalanine in the enzyme–substrate complex, a study of the inhibitory properties of N-substituted L-phenylalaninamides was undertaken (Table VIII). With the exception of the *N*-methylamide (LX), where double-reciprocal plots could not be obtained because of its poor inhibitory properties, all analogs listed were competitive inhibitors with respect to phenylalanine and, in cases examined, noncompetitive with respect to ATP. It is noted that substitution of a methyl group for an amide hydrogen of XVIII to give *N*-methyl-L-phenylalaninamide (LX) results in 857-fold decrease in binding. As the carbon chain of *N*-alkylphenylalaninamides is extended binding is gradually increased, the *N*-pentylamide (LXIV) binding some 41 times better than LX. A similar systematic increase in binding is observed upon incremental lengthening of the carbon chain of *N*-phenylalkylamides (LVI to LXX), which are uniformly better inhibitors than the *N*-alkyl derivatives. Optimum binding is achieved with the phenylpentylamide (LXX) which is about as potent an inhibitor as L-phenylalaninamide (XVII). When a carboxylate group is placed on the carbon chain of LXVII, as in L-phenylalanyl-L-phenylalanine (LXXII), inhibitory power is severely affected.

Discussion

The effects of systematic structural modifications of a substrate upon its affinity to an enzyme may be utilized to describe the critical intermolecular interactions responsible for binding and specificity. Using expected values for the magnitude of intermolecular forces in aqueous solution (Jencks, 1969), as well as considerations of the abruptness of topographical changes on protein surfaces, changes in inhibitory power which result from structural modifications may, with certain limitations, be related to the microenvironment of the binding site and the forces which lead to complexation. Inherent in an analysis of this type are a number of pitfalls which could easily result in misinterpretation of the binding forces involved. Foremost is the consideration of whether the inhibitors being compared are bound in an analogous

TABLE VII: Binding of the α-Amino Group of Phenylalanine and Analogs.

	$C_6H_5CH_2R$, R	K_i (mM)[a]
XLIX	CH_2CO_2H	9.7
L	$CHCO_2H$ | OH	2.2
LI	$C(=O)CO_2H$	7.6
LII	NH_2	2.0
LIII	$(CH_2)_2NH_2$	1.8
LIV	$CHCO_2H$ | $NHCH_3$	18
LV	$CHCO_2H$ | $NHCH_2C_6H_5$	*b*
LVI	$CHCO_2H$ | $CH_3NCH_2C_6H_5$	*b*
LVII	CH_2 | $NHCH_3$	6.0
LVIII	$CHCH_3$ | $NHCH_3$	8.0
LIX	$CHCH_3$ | $N(CH_3)_2$	9.5

[a] None of the analogs listed stimulated ATP–PP_i exchange at concentrations comparable to K_i or at maximum solubility. [b] No inhibition at maximum solubility (0.5 mM).

TABLE VIII: Binding Constants of L-Phenylalaninamides, $C_6H_5CH_2CH(NH_2)C(=O)R$.

	R	K_i (mM)	$-\Delta F$
XVIII	NH_2	0.035	6.32
LX	$NHCH_3$	30[a]	2.16
LXI	$NHCH_2CH_3$	6.07	3.14
LXII	$NH(CH_2)_2CH_3$	2.46	3.70
LXIII	$NH(CH_2)_3CH_3$	1.88	3.87
LXIV	$NH(CH_2)_4CH_3$	0.73	4.45
LXV	NHC_6H_5	0.20	5.25
LXVI	$NHCH_2C_6H_5$	0.82	4.38
LXVII	$NH(CH_2)_2C_6H_5$	0.45	4.75
LXVIII	$NH(CH_2)_3C_6H_5$	0.068	5.91
LXIX	$NH(CH_2)_4C_6H_5$	0.10	5.67
LXX	$NH(CH_2)_5C_6H_5$	0.045	6.17
LXXI	NH-β-naphthyl	0.41	4.80
LXXVIII	L-Phenylalanine	>5	

[a] Estimated from per cent inhibition.

manner to the substrate. In the present study, three criteria were used to provide this assurance. First, in accord with the random order of substrate addition to PRS (Santi *et al.*, 1971), analogs which bind to the amino acid binding site should be competitive inhibitors with respect to phenylalanine and noncompetitive with respect to ATP. Second, as will be shown later, analogs having an asymmetric carbon should demonstrate stereospecific inhibition of PRS, the more potent of the enantiomers being that which corresponds to the L-phenylalanine configuration. Third, structure–binding relationships are utilized to interrelate the various series of analogs; that is, structural modification of one series of analogs should produce parallel binding effects as the same modifications in another.

A further problem is whether observed binding energies of the analogs solely reflect primary interactions with the enzyme, or whether they include secondary effects resulting from complexation. Pertinent examples of secondary binding effects are energetically unfavorable conformational changes of the protein, distortion or strain of the inhibitor accompanying complexation, and binding of a subspecies of the inhibitor which is of higher energy than its ground state in solution. In such cases, the observed binding energy is less than that attributable to the primary interaction by the amount necessary to produce the conformational change, distortions or subspecies conversion. A related situation, where observed differences in binding may not solely be related to primary interactions of the variable substituents, could arise if interactions at one site of complexation result in perturbations of other binding loci.

The possible role of analogous effects in enzyme catalysis has recently been reviewed (Jencks, 1969), and there seems to be little reason to doubt their common occurrence. With regard to the present study, the important question is how these phenomena might affect interpretations of the differences in binding observed within a series of related analogs. If a secondary binding effect occurs to the same magnitude upon complexation of each inhibitor in a series, the relative positioning of the dissimilar groups with respect to the enzyme binding site will not be altered. Under this circumstance, the energy expended to produce the effect will be constant and the primary interactions of the dissimilar substituents will be manifested in the observed *differences* in the free energies of binding ($\Delta\Delta F$). On the other hand, if a particular substituent produces a unique secondary effect, $\Delta\Delta F$ will not serve to relate the primary interaction with other members of the series. Although it is clear that unique secondary binding effects could result in misleading interpretations of binding forces, it is difficult to devise direct experimental tests for their occurrence. For this reason, it is important that the model of a binding site which is constructed by this method is based on the binding properties of a large number of competitive inhibitors. Unique binding effects will often be manifested as deviations in linear free energy relationships correlating structure with binding. In addition, if a proposed interaction is consistent with the binding properties of a number of disparate groups, the probability of misinterpretation resulting from a unique secondary binding effect is greatly reduced.

The mechanism by which an aa-tRNA synthetase distinguishes 1 amino acid from the other 19 must reside, directly or indirectly, in the region of the enzyme which proximates the side chain of the amino acid in the enzyme–substrate complex. As indicated by the binding properties of phenyl-substituted phenylalanines, phenylalaninols, and amphetamines (Tables III and IV), PRS is highly discriminating for the unsubstituted benzene ring of the natural substrate.

Although it is difficult to ascertain exactly what interactions are involved, the correlations which may be made between the size of substituents and the affinity to PRS indicate that steric effects largely determine the specificity in this region. The substituents examined also exert different electronic effects upon the phenyl ring, but these cannot be related to binding. In this regard it is noted that the isoteric CF_3 and CH_3 groups, which differ significantly in the electronic effects exerted on the benzene ring, produce identical inhibitory effects (Table V). Within the series investigated, meta substituents appear to be slightly better tolerated by PRS than those at the ortho or para positions. It is also interesting that the *p*-hydroxy group appears to be more detrimental to binding than the other substituents examined and may, in fact, prevent complexation to the active site. This is not surprising since PRS must distinguish L-tyrosine from the natural substrate with perfect fidelity. The previously discussed steric requirements for binding indicate that even an unsolvated hydroxyl group would be sufficiently large to prohibit significant complexation at concentrations of tyrosine found *in vivo*. In addition, if the cavity of PRS is properly described as a hydrophobic pocket with rigid steric requirements, binding of L-tyrosine or its analogs would require energetically unfavorable removal of one or more molecules of water from the hydration sphere surrounding the phenolic hydroxyl group.

From the results presented in Table VII it is apparent that the α-amino group of phenylalanine is of major importance for complexation to PRS. Replacement by hydrogen, oxo, hydroxyl, acylamido (Conway *et al.*, 1962), or alkylamino results in substantial losses in binding as well as complete elimination of ATP–PP_i exchange stimulation. Under the assay conditions used, the amino group of phenylalanine and most analogs examined is present in solution almost entirely as its conjugate acid. If the species which binds to PRS is the free amine, the observed free energy of binding would be decreased by the amount necessary to dissociate the protonated amine. Since, in many cases, this would correspond to unreasonably low dissociation constants, it is probably true that the amino group interacts as its conjugate acid, perhaps with a corresponding anionic site of the enzyme. Some support for this is provided by the large loss in binding observed upon substitution of the CH_3 group of amphetamine by CF_3. Since the carboxylate binding site of PRS will tolerate a large variety of substituents (*vide infra*), the effect is probably a result of the large decrease in the basicity of the amine caused by the α-CF_3 group so that only a small fraction is present as the conjugate acid under the assay conditions. In addition, the observation that the N-methylated analogs of phenylalanine are poorly bound to PRS indicates that rigid steric requirements also exist in this region of the enzyme, and that the tolerance for groups larger than hydrogen is low.

As with other aa-tRNA synthetases (Stern and Mehler, 1965; Mitra and Mehler, 1967; Loftfield and Eigner, 1966), the optimum separation between the side chain and amino binding points of phenylalanine analogs is that which is found in the natural substrate. Using 2-phenylethylamine as reference, lengthening or shortening the chain separating these binding loci by one methylene group results in approximately 20-fold loss in binding to PRS. *A priori*, these modifications might have been expected to be more detrimental to binding than the results indicate. The possibility that specific binding of the amino group to PRS is unimportant is not in accord with the deleterious effects which result from the many modifications of this group which have been performed; furthermore, as will be discussed later, the high degree of stereoselectivity which is observed with PRS requires that the α-amino group be rigidly bound to the enzyme. It would appear more reasonable to believe that conformational changes of the enzyme may occur at the expense of binding energy, which permits interaction of the amino group with the cognate site of PRS. Alternatively, or in conjunction with the above, molecular models show that the homologous series of phenylalkylamines examined have a number of possible conformations in which the phenyl and amino groups possess similar relative positions. Thus, the decreases in binding observed with benzylamine and 3-phenylpropylamine may in part reflect the binding of energetically unfavorable conformations of these analogs, or steric interactions between the carbon chain and the enzyme.

The α-amino group appears to be a major binding locus for a number of aa-tRNA synthetases and is absolutely essential for substrate activity (Calendar and Berg, 1966; Papas and Mehler, 1970; Conway *et al.*, 1962; Owens and Bell, 1970). Since, with the exception of proline, the primary amino group is found in all of the ligase substrates, it cannot in any obvious manner serve to select the proper amino acid; however, in view of its rudimentary role in binding and the sensitivity of binding toward structural modifications, the primary amino group must certainly be one of the common recognition sites for this group of enzymes. An exception to the above may be found in the case of Pro-tRNA synthetase (Papas and Mehler, 1970) where the secondary amino group serves as a major basis for distinguishing proline from the other 19 amino acids. As will be discussed below, we envisage that the primary amino group and the benzene ring of L-phenylalanine serve as major anchor points to direct the carboxylate group and α hydrogen into the necessary positions for catalysis and stereospecificity.

Comparison of the inhibitory powers of a number of α-substituted 2-phenylethylamines permits a description of the sites of PRS which proximate the α-hydrogen and carboxylate moiety of L-phenylalanine. As shown with molecular models, tight and uniform binding of the phenyl and amino groups of these analogs severely restricts the rotation of the remainder of the molecule. By using the proper enantiomer, an α substituent may be selectively projected into either region of the enzyme and the differences in free energies of binding may be related to the intermolecular interactions of the dissimilar α substituents. In the ensuing discussion, the carboxylate and α-hydrogen binding regions of PRS are referred to as regions A and B, respectively, and 2-phenylethylamine is used as an arbitrary reference to which binding of other analogs is compared. For descriptive purposes, it is assumed that hydrogen atoms in regions A and B neither contribute to, nor detract from, binding to PRS.

Using this model, if K_m is taken as a measure of the dissociation constant of L-phenylalanine, the binding energy contributed by the carboxylate group is calculated to be only 0.3 kcal/mole. Interestingly, when the negatively charged carboxylate group is substituted by neutral groups which are more hydrophobic, binding energy is gained. The carboxamide group of L-phenylalaninamide provides 0.6 kcal/mole in binding energy, and hydroxymethyl and methyl groups provide 1.68 and 1.27 kcal/mole, respectively. This is further substantiated by comparison of Tables III and V; in every case examined, replacement of the carboxylate of the nonsubstrate β-arylalanines by hydroxymethyl or methyl groups results in higher affinity of the analog for PRS. The hydroxyl group of the phenylalaninols may serve as a hydrogen bond acceptor or donor but the binding energy which may be gained

from such interactions in water is small (Jencks, 1969) and, as must certainly be the case with the amphetamines, hydrophobic forces and specific solvation are probably responsible for the increased binding.

The carboxylate anion is of course heavily solvated in aqueous media and, *a priori*, one would expect that the proximate region of the enzyme in the enzyme–substrate complex would be complementary in polarity and solvation. If this region of the enzyme is polar, then occupation by poorly solvated or hydrophobic groups would be accompanied by a compensatory loss in binding energy. Conversely, if the site is in part hydrophobic, then binding of the solvated carboxylate of the substrate to this site would be an energetically unfavorable process. Of course, complete transfer of the polar carboxylate from aqueous solution to a hydrophobic region of the enzyme is energetically unfeasible. However, within the boundaries of the energy differences we observe, it is not unreasonable to suggest that a partial transfer occurs; this might involve the judicious removal or reorientation of only a single water molecule from the hydration sphere of the carboxylate when bound to a localized nonpolar region of the binding site. Why the enzyme would evolve in this seemingly inefficient manner provides an interesting point for speculation. It is well known (see, for example, Jencks, 1969, and Bruice, 1970) that the rates of certain types of nucleophilic displacement reactions may be greatly enhanced by desolvation of the ground and transition states. In analogy, it has been conjectured that due to the localized polarity of the active site of an enzyme, solvation of bound substrates and transition states may be manipulated to maximize catalysis, often at the expense of binding energy. For the case in point, partial desolvation of the carboxylate anion of L-phenylalanine in the enzyme–substrate complex would be expected to increase the ground state free energy and thus reduce the free energy of activation leading to aminoacyladenylate formation; in essence, although binding energy is lost, the desolvated carboxylate will be a more powerful nucleophile than the completely hydrated species.

A number of workers (Cassio *et al.*, 1967; Calendar and Berg, 1966; Owens and Bell, 1970; Papas and Mehler, 1970) have observed that substitution of the carboxylate groups of a variety of amino acids by neutral, relatively hydrophobic groups results in derivatives which are potent inhibitors of their cognate aa-tRNA synthetases. Using similar arguments as presented above, it is tempting to suggest that a common feature of many of these may involve the ability to desolvate the carboxylate anion of the amino acid in the enzyme–substrate complex. Through this, a general mechanism is provided by which catalysis may be facilitated at the expense of binding energy.

The aa-tRNA synthetases may possess varying degrees of stereospecificity for amino acids with respect to both binding and substrate properties. For example, D-tyrosine will stimulate ATP–PP_i exchange and become esterified with $tRNA^{Tyr}$, albeit with higher K_m values than with the L isomer, in the presence of purified Tyr-tRNA synthetases from *E. coli* or *Bacillus subtilis* (Calendar and Berg, 1966). With Val-tRNA synthetase, only the L enantiomorphs of amino acid substrates will support ATP–PP_i exchange (Owens and Bell, 1970), but the D enantiomorphs are good competitive inhibitors with respect to L-valine. In contrast, very high concentrations of D-proline do not inhibit Pro-tRNA synthetase (Papas and Mehler, 1970).

The stereospecificity of PRS is absolute with respect to both substrate and binding properties. D-Phenylalanine does not stimulate ATP–PP_i exchange and shows an estimated K_i which is some 170 times greater than K_m for the L enantiomorph; it is noted that because of the poor inhibitory properties of the D enantiomorph, the type of inhibition produced could not be ascertained and it is possible that it does not bind to the active site. Using the previously discussed model, it is possible to define better the mechanism and extent of stereospecificity exhibited by PRS. For this analysis, differences in the free energy of binding which result upon projection of methyl and hydroxymethyl groups into the region of PRS which is normally occupied by the α hydrogen of L-phenylalanine are compared to the reference standard β-phenylethylanine and to analogs in which the substituents are projected into the carboxylate binding region (region A) of the enzyme. The binding energy gained upon substitution of a hydroxymethyl group for the hydrogen in region A, as in L-phenylalaninol (XIX), amounts to 1.68 kcal, and substitution of a hydroxymethyl group for the hydrogen β-phenylethylamine in region B (D-phenylalaninol, XLII) results in a loss of 1.68 kcal of binding energy. Similarly, a methyl group projected into region A (L-amphetamine, XX) contributes 1.27 kcal to binding, whereas the same group in region B (D-amphetamine, XLIII) detracts 0.44 kcal in binding energy. Since the above enantiomorphs do not differ in any way except the direction in which the α substituents are projected, the values obtained should reflect specific interactions of the methyl and hydroxymethyl groups with the defined regions of the enzyme and the free energies of binding should be additive. Thus, the binding energy of α,α-dimethyl-2-phenylethylamine (XLIV), which projects methyl groups into both regions A and B, might be expected to exceed that of 2-phenylethylamine by 0.83 kcal/mole. However, XLIV shows a loss of 1.0 kcal/mole in binding energy. A similar calculation would predict that (*R*)(+)-α-methylphenylalaninol (XLV), where CH_2OH is in region B and CH_3 occupies region A, would only result in a decrease of 0.4 kcal/mole of binding energy as compared to 3-phenylethylamine. Experimentally, the loss in binding energy is much greater than this and amounts to some 1.27 kcal/mole. If the stereochemistry of these substituents is reversed, an increase of 1.22 kcal/mole in binding energy over β-phenylethylamine is expected, which is not in agreement with the derived value for (*S*)(−)-α-methylphenylalaninol which indicates a loss of 0.8 kcal/mole.

The fact that linear free-energy relationships are not followed indicates that projection of a methyl or hydroxymethyl group into region B results in one of the aforementioned secondary binding effects. This might occur if the interaction of region B with the α hydrogen of L-phenylalanine is sterically close, and occupation by larger groups resulted in disruption of favorable interactions between the methyl or hydroxymethyl groups and the carboxylate binding region (region A). This effect might also manifest itself in deformations of the inhibitor or enzyme which prevent optimum interactions of the phenyl and amino groups with their cognate sites of PRS.

The model for stereospecificity of binding may also be used to interpret and predict aspects of substrate specificity. L-Phenylalanine derivatives which possess an α substituent larger than hydrogen should be poorly bound nonsubstrates, since the energetically unfavorable projection of bulky substituents into region B will not permit the proper juxtapositioning of the carboxylate in region A for catalysis. This is borne out by the observation that α-methyl-L-phenylalanine does not stimulate ATP–PP_i exchange and is very poorly bound to PRS. D-Phenylalanine is a poor-binding nonsub-

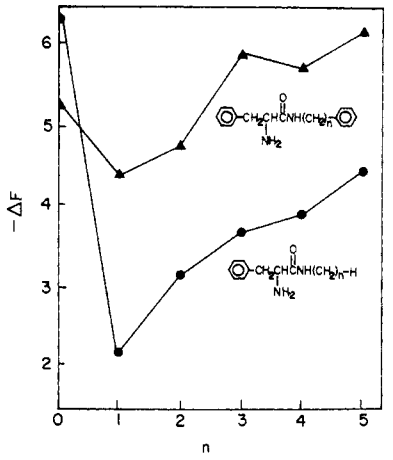

FIGURE 1: Plot of negative free energy of binding ($-\Delta F$) as a function of carbon chain length, n, of alkyl- (●) and phenylalkyl- (▲) amides of L-phenylalanine.

strate since projection of the carboxylate in the catalytic site (region A) would require placement of the protonated amino group in the sterically confined region B and accompanying loss of the critical interactions which occur between PRS and the α-amino group. It is also apparent from the above that the inhibition produced by D,L mixtures of the analogs is due solely to the L enantiomorph, and true K_i values of racemic mixtures may be obtained by dividing the observed dissociation constants by two.

Although L-phenylalaninamide is a good competitive inhibitor of PRS, addition of a methyl group on the amide nitrogen results in an approximate 1000-fold increase in dissociation constant. This 4.2-kcal decrease in binding energy is much too large to be attributed to the loss of any single intermolecular interaction and is best attributed to a very unfavorable steric interaction of the methyl group within the E·I complex. The affinities of the *N*-alkyl- and *N*-phenylalkylamides of phenylalanine for PRS increase as the carbon chains are lengthened. The parallel binding profiles (Figure 1) observed for these two series provide support that the alkyl and phenylalkyl groups are bound in an analogous manner. Calculated differences in the free energy of binding ($\Delta\Delta F$) between the *N*-methylamide and homologous *N*-alkylamides provide estimates ranging between 0.2 and 1.0 kcal/mole for the contribution of each methylene group after the initial detrimental interaction. Similarly, $\Delta\Delta F$ comparisons between alkyl- and phenylalkylamides having the same chain length indicate that the benzene ring provides an additional 1.75–2.25 kcal/mole in binding. It is also noted that placement of a polar group in this region (*viz.*, carboxylate of LXXII) results in large losses in binding. We currently interpret these results as evidence for the existence of a diffuse hydrophobic region adjacent to the site of PRS which proximates the carboxylate of L-phenylalanine. As the hydrocarbon chain of alkyl- and aralkylamides is increased, their interactions with this region of the enzyme counteract the detrimental effect of the sp^3-hybridized carbon attached to the amide nitrogen. These interactions do not provide the estimated maximal 0.8–1.0 kcal/mole per methylene or 5 kcal/mole per benzene binding energy which might be expected (Jencks, 1969) from complete transfer into a hydrophobic region of the enzyme. Nevertheless, sufficient binding energy is obtained that the phenylpentylamide completely compensates for the unfavorable interaction and binds as well as the parent phenylalaninamide.

In conclusion, the binding properties of a large number of competitive inhibitors have been correlated and analyzed in order to construct a model of the phenylalanine binding site of PRS. Although numerous difficulties and shortcomings are inherent in this approach, the description is adequate to permit an assessment of intermolecular interactions responsible for binding and recognition of L-phenylalanine. Such information should be useful for a variety of purposes, ranging from studies of enzyme–substrate interactions to those concerned with *in vivo* effects of inhibition of a particular activating enzyme. Work in progress involves the utilization of these results for affinity labeling and chromatography of PRS, as well as comparative studies of the amino acid binding sites of various ligases. Hopefully, these investigations will uncover common features of this group of enzymes, as well as basic differences, which might permit a better understanding of the molecular basis of substrate binding and recognition.

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