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Evolutionary Aspects of Accuracy of Phenylalanyl-tRNA Synthetase. A Comparative Study with Enzymes from *Escherichia coli*, *Saccharomyces cerevisiae*, *Neurospora crassa*, and Turkey Liver Using Phenylalanine Analogues†

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ABSTRACT: The phenylalanyl-tRNA synthetases from *Escherichia coli*, *Saccharomyces cerevisiae*, *Neurospora crassa*, and turkey liver activate a number of phenylalanine analogues (tyrosine, leucine, methionine, *p*-fluorophenylalanine, β-phenylserine, β-thien-2-ylalanine, 2-amino-4-methylhex-4-enoic acid, mimosine, *N*-benzyl-L- or *N*-benzyl-D-phenylalanine, and ochratoxin A), as demonstrated by K_m and k_{cat} of the ATP/PP_i pyrophosphate exchange. Upon complexation with tRNA, the enzyme–$tRNA^{Phe}$ complexes show a significantly increased initial discrimination of these amino acid analogues expressed in higher K_m and lower k_{cat} values, as determined by aminoacylation of $tRNA^{Phe}$-C-C-A(3′NH_2). The overall accuracy is further enhanced by a second discrimination, a proofreading step. The strategies employed by the enzymes with respect to accuracy differ. Better initial discrimination in the aminoacylation and less elaborated proofreading for the *E. coli* enzyme can be compared to a more efficient proofreading by other synthetases. In this way the comparatively poor initial amino acid recognition in the case of the *S. cerevisiae* and *N. crassa* enzymes is balanced. The extent of initial discrimination is therefore inversely coupled to the hydrolytic capacity of the proofreading. A striking difference can be noted for the proofreading mechanisms. Whereas the enzymes from *E. coli*, *S. cerevisiae*, and *N. crassa* follow the pathway of posttransfer proofreading, namely, enzymatic hydrolysis of the misaminoacylated tRNA, the turkey liver enzyme uses tRNA-dependent pretransfer proofreading in the case of natural amino acids. In spite of the same subunit structure and similar molecular weight, the phenylalanyl-tRNA synthetases from a prokaryotic and lower and higher eukaryotic organisms show obvious mechanistic differences in their strategy to achieve the necessary fidelity.

Aminoacyl-tRNA synthetases esterify an amino acid with its cognate tRNA with an error rate of smaller than 10^{-4} (Loftfield & Vanderjagt, 1972). In fact, a specificity of smaller than 10^{-5} is observed as a result of a proofreading step subsequent to initial binding and activation of the amino acid (Cramer et al., 1979; von der Haar et al., 1981). For the proofreading capacity of the aminoacyl-tRNA synthetases, different mechanistic interpretations are given in the literature. It was suggested by Hopfield that specificity is enhanced by kinetic proofreading via the preferential dissociation of the wrong aminoacyladenylate (Hopfield, 1974; Hopfield et al., 1976). This pathway has been questioned by von der Haar (1977), Fersht (1977b), and Igloi et al. (1978). von der Haar and Cramer have suggested chemical proofreading as a corrective step after transfer of the amino acid to the tRNA, which leads to emzymatic hydrolysis of the ester linkage between the tRNA and the wrong amino acid (von der Haar & Cramer, 1975, 1976). A similar scheme for the discrimination between cognate and noncognate substrates depending on the relative rates of synthesis and hydrolysis was developed by Fersht, who introduced the double-sieve model (Fersht, 1977a; Tsui & Fersht, 1981). Generalization of such mechanistic descriptions must be done with caution, since synthetases from specific organisms are necessarily used for these investigations. Discrepancies in the mechanistic interpretations are possible because it is not sufficiently well established that the proofreading mechanisms of specific synthetases from different organisms are identical. An indication of different behavior is also provided by the example of different extent of activation of some nonprotein amino acids that are produced only in certain plants [e.g., Lea & Norris (1977)]. It should also be noted that there are differences in the catalytic mechanisms of other enzymes with respect to evolution, for example, the fatty acid synthetase (Lynen, 1980).

In this paper, the interaction of 11 phenylalanine analogues with the phenylalanyl-tRNA synthetase from *Escherichia coli*, *Saccharomyces cerevisiae* (yeast), *Neurospora crassa*, and turkey liver was studied by analysis of ATP/PP_i pyrophosphate exchange, aminoacylation of yeast $tRNA^{Phe}$-C-C-A and $tRNA^{Phe}$-C-C-A(3′NH_2), and AMP production during aminoacylation of $tRNA^{Phe}$-C-C-N. From the studies, a comprehensive picture emerges of how the phenylalanyl-tRNA

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synthetases from different organisms achieve the required fidelity. It seems that proofreading is a late invention of evolution that becomes more refined in the higher branches of the evolutionary tree.

Experimental Procedures

Materials. $tRNA^{Phe}$-C-C amd $tRNA^{Phe}$-C-C-A were isolated by the procedure of Schneider et al. (1972) from unfractionated bakers' yeast tRNA (Boehringer Mannheim, FRG). Incorporation of 3′-deoxy-3′-aminoadenosine 5′-phosphate into $tRNA^{Phe}$-C-C was performed with tRNA nucleotidyltransferase (EC 2.7.7.25), which was kindly provided by Dr. H. Sternbach, as described (Sprinzl & Sternbach, 1979). Phenylalanyl-tRNA synthetase (EC 6.1.1.20) from *E. coli*, isolated according to Holler et al. (1975), was kindly provided by Dr. E. Holler, Regensburg, FRG. The homogeneous enzyme was isolated from yeast by use of the $tRNA^{Phe}$-induced solubility shift in salting-out chromatography as described by von der Haar (1978), from *N. crassa* by affinity elution (von der Haar, 1973), and from turkey liver cytoplasm over 5000-fold essentially as described for the mitochondrial enzyme by Gabius & Cramer (1982). Proteinase K (EC 3.4.21.14) was from Boehringer (Mannheim, FRG). ^{14}C-Labeled amino acids of the specific activity of 521 mCi/mmol, [^{14}C]ATP of the specific activity of 55 mCi/mmol, and [^{32}P]pyrophosphate of the specific activity of 11.3 mCi/mmol were purchased from Radiochemical Centre (Amersham, England). Phenylalanine (L and D) was obtained from Merck (Darmstadt, FRG); tyrosine, methionine, leucine, *p*-fluorophenylalanine, β-phenylserine, and ochratoxin A (7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3(*R*)-methylisocoumarin linked by its 7-carboxyl group to L-phenylalanine) were from Sigma (Munich, FRG), β-thien-2-ylalanine and mimosine [β-*N*-(3-hydroxy-4-pyridone)alanine] were purchased from Serva (Heidelberg, FRG), 2-amino-4-methylhex-4-enoic acid was a gift from Dr. L. Fowden (London), and *N*-benzyl-L- or *N*-benzyl-D-phenylalanine were synthesized from nonradioactive and [^{14}C]phenylalanine. Other chemicals were commercially available analytical grade.

ATP/PP_i Pyrophosphate Exchange. The incorporation of [^{32}P]pyrophosphate into ATP catalyzed by the phenylalanyl-tRNA synthetases was monitored as described (Igloi et al., 1979). The incubation mixture contained 1.5 mM [^{32}P]pyrophosphate (sp act. ~3000 cpm/nmol) and varying concentrations of amino acid in a total volume of 0.1 mL at 37 °C. The reaction was initiated by the addition of 4–6 μg of synthetase. Kinetic parameters were obtained by double-reciprocal plots (Lineweaver & Burk, 1934) with computerized linear regression.

Aminoacylation. The aminoacylation of unfractionated tRNA was performed at 37 °C in 0.1 mL of solution containing 150 mM tris(hydroxymethyl)aminomethane (Tris-HCl) (pH 7.65), 150 mM KCl, 20 mM $MgSO_4$, 2 mM ATP, 50 μg of bovine serum albumin, 100 nL of β-mercaptoethanol, 1 mg of unfractionated tRNA, and 0.02 mM ^{14}C-labeled amino acid (1 nmol = 70000 cpm). The incorporation of ^{14}C-labeled amino acid was measured as described (von der Haar & Gaertner, 1975). $tRNA^{Phe}$-C-C-A and $tRNA^{Phe}$-C-C-A(3′NH_2) were aminoacylated under conditions described above but with 0.05 mM ^{14}C-labeled amino acid and 5.7 μM tRNA. The detection of the transfer of nonradioactive amino acid to $tRNA^{Phe}$-C-C-A(3′NH_2) by the method of back-titration has been described (Igloi et al., 1977). With 5-min preincubation and 1.2 μg of enzyme, transfer of nonradioactive amino acids to $tRNA^{Phe}$-C-C-A was also measured by the method of back-titration. So that one could obtain the kinetic parameters for aminoacylation of $tRNA^{Phe}$-C-C-A(3′NH_2) with nonradioactive analogs, a high excess of [^{14}C]phenylalanine was added to the reaction mixture after varying incubation times, which resulted in aminoacylation of the residual $tRNA^{Phe}$ with phenylalanine. As a control, these results were confirmed with available ^{14}C-labeled amino acids. Again, the data were processed with a computer program for linear regression. For product analysis in special cases, aminoacylated $tRNA^{Phe}$-C-C-A and $tRNA^{Phe}$-C-C-A(3′NH_2) were isolated according to Sprinzl & Sternbach (1979). Hydrolysis was performed with alkali or proteinase K, and the amino acids were separated by ascending chromatography on poly(ethylenimine) (PEI) plates with butanol–acetic acid–water (4:1:1) as solvent. The stoichiometry of Phe-$tRNA^{Phe}$ formation was measured in the standard buffer with 35 μM $tRNA^{Phe}$ and 1 μM enzyme according to Fasiolo & Fersht (1978).

Transient Formation of ^{14}C-Labeled Aminoacyl-$tRNA^{Phe}$. The enzyme–^{14}C-labeled aminoacyl-AMP complex was formed in situ at 0 °C in the standard buffer system in the presence of 0.5 unit of inorganic pyrophosphatase (EC 3.6.1.1), 2.5 mM ATP, and 200 μM ^{14}C-labeled amino acids. After a periodically monitoring by nitrocellulose filter assays, the reaction was started by addition of $tRNA^{Phe}$ (7 μM) and nonradioactive amino acid (1 mM) to the solution containing the enzyme–^{14}C-labeled aminoacyl-AMP complex (2.8 μM). Aliquots were removed at 10-s intervals, spotted onto paper filter disks (Whatman, 3 MM), and immediately quenched with trichloroacetic acid.

Attempts to transfer ^{14}C-labeled amino acids from the isolated enzyme–^{14}C-labeled aminoacyl-AMP complex (which had been freed from excess ligand by gel filtration) to unfractionated tRNA were made under standard conditions at pH 7.65 by the following procedures: (a) tRNA was added to the complex, and the mixture was quenched with trichloroacetic acid to collect any aminoacylated tRNA. (b) The above was repeated, but immediately, yeast aminoacyl-tRNA synthetases (1 μM) were added in order to trap any ^{14}C-labeled aminoacyl-AMP (tyrosine, leucine, methionine) that dissociated from the enzyme. In this case, the unlabeled natural amino acid (1 mM) and alkaline phosphatase (EC 3.1.3.1, 0.4 unit/mL) were added to suppress any aminoacylation of tRNA due to residual ATP and ^{14}C-labeled amino acid that is released by the hydrolysis of [^{14}C]aminoacyl-AMP. (c) The same procedure as (a) was used, but instead of being quenched, the mixture was filtered through nitrocellulose disks to detect any remaining enzyme–^{14}C-labeled aminoacyl-AMP complex.

AMP Production during Aminoacylation. Production of AMP under aminoacylation conditions, the AMP/PP_i independent hydrolysis, was determined at 37 °C in an assay mixture containing 150 mM Tris-HCl (pH 7.65), 150 mM KCl, 20 mM $MgSO_4$, 1 mM amino acid, 0.5 mM [^{14}C]ATP (with a sp act. of 55 mCi/mmol), and 2.5 μM tRNA in a total volume of 50 μL. The reaction was initiated by the addition of 10–25 μg of enzyme. Aliquots of 1 μL containing approximately 30000 cpm are removed after certain time intervals (typically 0, 5, 10, 20, and 30 min) and processed as described by von der Haar & Cramer (1976).

Check of the Purity of Enzyme Preparations and Amino Acids. Aminoacylation tests with bulk tRNA were carried out with different ^{14}C-labeled amino acids to ensure the homogeneity of the enzyme sample. Only enzyme preparations free of any other activity were used in this study. Contamination of any other amino acid by phenylalanine was investigated by studying the isotope-dilution effects of the amino acid at 1 mM concentration on aminoacylation of

$tRNA^{Phe}$-C-C-A in the presence of [^{14}C]phenylalanine.

Calculation of Error Rates. The relevant kinetic parameter for calculating error rates in comparison of the data from the pyrophosphate exchange and aminoacylation of $tRNA^{Phe}$-C-C-A(3′NH_2), summarized in Table I, is k_{cat}/K_m since the relative reaction velocity of two substrates A and B competing for the same enzyme is given by (Fersht, 1977a)

$$V_A/V_B = (k_{cat}/K_M)_A C_A/[(k_{cat}/K_m)_B C_B] \quad (1)$$

From amino acid pool data given by Holden (1962), Raunio & Rosenqvist (1970), and Buttery & Rowsell (1974), the ratio of phenylalanine to other natural amino acids is known. Thus the upper limit of the error rate can be calculated. The difference in binding energy of amino acids containing different side chains relative to phenylalanine ($\Delta\Delta G_B$) for physicochemical considerations is obtained from the k_{cat}/K_m values as described by Fersht (1977a):

$$(k_{cat}/K_m)_A/(k_{cat}/K_m)_B = \exp[-\Delta\Delta G_B/(RT)] \quad (2)$$

Results

Selection of Methods and Eleven Phenylalanine Analogues. An indication of specificity in the recognition of amino acids by the enzymes was given by the kinetic parameters of activation of different phenylalanine analogues, as monitored by the ATP/PP_i exchange in the absence of $tRNA^{Phe}$. Measurements of the extent and the kinetic parameters for the aminoacylation of $tRNA^{Phe}$-C-C-A and $tRNA^{Phe}$-C-C-A-(3′NH_2) (where the potentially labile ester bond of the aminoacyl-tRNA is replaced by a stable amide link) helped to quantify the transfer of misactivated amino acids to $tRNA^{Phe}$. The observation of transient formation of aminoacyl-$tRNA^{Phe}$-C-C-A with noncognate amino acids served as a control to test whether the transfer of an amino acid to $tRNA^{Phe}$-C-C-A(3′NH_2) is part of the normal catalytic cycle. Analyses of the rate and extent of AMP production under aminoacylation conditions are further indicators for misactivation and proofreading mechanism. The role played by the tRNA in AMP production that is nonstoichiometric with respect to aminoacylation and indicative of proofreading can be determined with $tRNA^{Phe}$s modified at the accepting 3′-terminal adenosine. Following the outline given by Igloi et al. (1979) to unequivocally identify the misactivation of the tested phenylalanine analogues, problems of contamination in either the enzyme preparation or in the amino acid that can lead to misinterpretations of the data were shown to be negligible.

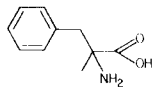
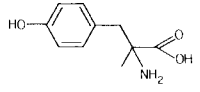
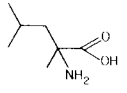
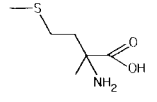
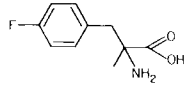
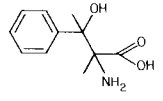
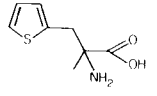
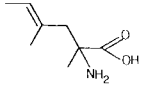
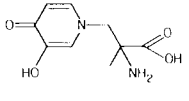
The analogues used can be classified as follows. (a) Natural amino acids: These include tyrosine, leucine, and methionine. It is known that they are transferred to $tRNA^{Phe}$-C-C-A-(3′NH_2) by the yeast enzyme (Igloi et al., 1978) and that they are very close to phenylalanine in a "similarity ring" according to the probability matrix of substitution (Argyle, 1980). (b) Analogues with ring substitution: *p*-Fluorophenylalanine with its altered ring electron distribution is nevertheless incorporated into protein of various organisms (Wheatley, 1978). Systematically, tyrosine would also belong to this group. (c) Analogues with different ring size: β-Thien-2-ylalanine, an analogue with a smaller ring size, is incorporated into protein and enhances proteolysis in *E. coli* (Janeček & Rickenberg, 1964; Pine, 1967). Mimosine, a nonprotein amino acid from *Leucaena leucocephala*, is activated in the pyrophosphate exchange by the *Phaseolus aureus* phenylalanyl-tRNA synthetase and the enzyme from other mimosine-producing plants (Smith & Fowden, 1968) and acts as growth inhibitor in *E. coli*, *P. aureus*, and rat (Fowden et al., 1967). (d) Analogues with only partial ring structure: An analogue, where the ring is opened but the moiety still planar, is 2-amino-4-methylhex-4-enoic acid, a nonprotein amino acid from *Aesculus californica*. It is activated in pyrophosphate exchange by enzymes of different Aesculus species and *P. aureus* (Anderson & Fowden, 1970). Systematically, leucine and methionine should also belong to this group, although the amino acid side chains contain no double bond to force coplanarity. (e) Analogues with substitution of the C_β atom: β-Phenylserine is incorporated into *E. coli* protein (Janeček, 1967). (f) Analogues with substituents on the α-NH_2 group: Three analogues have been chosen with substituents on the α-NH_2 group. *N*-Benzyl-L-phenylalanine exhibits no inhibition of the pyrophosphate exchange with *E. coli* enzyme; however, no indication had been given that it is a substrate (Santi & Danenberg, 1971). Therefore, *N*-benzyl-L- and *N*-benzyl-D-phenylalanine were selected. An analogue with an even larger substituent on the amino group, an isocoumarin moiety, is ochratoxin A from *Aspergillus ochraceus*. Ochratoxin A inhibits the protein synthesis of Gram-positive prokaryotic and yeast systems by competition with phenylalanine in the phenylalanyl-tRNA synthetase catalyzed reaction (Creppy et al., 1979).

Activation of Phenylalanine Analogues. The phenylalanyl-tRNA synthetases activate, in addition to phenylalanine, all tested analogues, as summarized in Table I. Since the properties of the *N. crassa* enzyme and the *S. cerevisiae* (yeast) enzyme are nearly identical, only the data for the yeast enzyme are included. In general, the reactions with noncognate amino acids are characterized by lower relative rates (compared with phenylalanine) and higher K_m values. The relative specificity against the noncognate amino acids, expressed as V_{Phe}/V_{Xxx}, is generally highest for the *E. coli* enzyme. In the case of discrimination against for example leucine (with respect to in vivo amino acid concentrations), the specificities are 7400:1, 360:1, and 7:1 for the *E. coli*, yeast, and turkey liver enzyme, respectively. However, this level of discrimination alone is clearly too low to ensure a high fidelity of protein synthesis and probably only reflects selection for the correct substrate on the basis of binding energy in the absence of tRNA.

Kinetic Data for Aminoacylation of $tRNA^{Phe}$-C-C-A-(3′NH_2) with Phenylalanine Analogues. If an enzyme–tRNA complex is used, the changes in the kinetic parameters indicate that the binding of the amino acid is much different with the binary enzyme–tRNA complex than with the free enzyme (Table I). The selection specificity is improved for all enzymes and analogues tested (with the exception of *p*-fluorophenylalanine and β-thien-2-ylalanine in the *E. coli* enzyme). For example, in the case of the yeast enzyme and the natural noncognate amino acids tyrosine, leucine, and methionine, the selection specificity rises from 98, 148, and 152 to 415, 27 000, and 23 000. These values, calculated according to eq 1 (assuming equal concentrations of phenylalanine and the analogue), are experimentally confirmed by measurement of the excess of analogue necessary to produce a 50% inhibition of phenylalanine aminoacylation. This excess is equivalent to the calculated ratio. Unfortunately, there is no detectable transfer of mimosine and ochratoxin A to tRNA. Therefore, it is difficult to speculate on effects in protein synthesis by the amino acid that may account for their growth-inhibiting or cytostatic-cytotoxic effects.

Aminoacylation of $tRNA^{Phe}$-C-C-A and $tRNA^{Phe}$-C-C-A-(3′NH_2) with Phenylalanine Analogues. After preincubation of the enzymes with nonradioactive amino acid and $tRNA^{Phe}$-C-C-A or $tRNA^{Phe}$-C-C-A(3′NH_2), the amount of residual nonaminoacylated tRNA is determined by the extent

Table I: Substrate Properties of the Tested Amino Acids for Phenylalanyl-tRNA Synthetase from *E. coli* (*E.c.*), Yeast (*S.c.*), or Turkey Liver (t.l.). The First Line Always Gives Data for Pyrophosphate Exchange; the Second Line, Data for Aminoacylation of tRNA-C-C-A(3′NH_2)

amino acid	K_m (μM)			k_{cat} (min^{-1})			V_{Phe}/V_{Xxx} [a]			V_{Phe}/V_{Xxx} [b]			$\Delta\Delta G_B$ (kJ/mol)		
	E.c.	*S.c.*	t.l.	*E.c.*	*S.c.*	t.l.	*E.c.*	*S.c.*	t.l.	*E.c.*	*S.c.*	t.l.	*E.c.*	*S.c.*	t.l.
phenylalanine (Phe)	3.4	33	29	645	712	355									
	2	9.4	13	108	255	30									
tyrosine (Tyr)	1 850	1 400	790	284	312	258	1 200	98	37	81 000	200	39	18.3	11.8	9.3
	1 700	1 000	0	17	65	0	5 400	415	0	370 000	1 600		22.1	15.5	
leucine (Leu)	250	170	229	109	25	190	435	148	15	7 400	360	7	15.6	12.8	7.0
	10 000	14 000	0	10	14	0	54 000	27 000	0	920 000	65 000		28.1	26.3	
methionine (Met)	410	210	237	182	30	196	425	152	15	10 000		12	15.6	12.9	7.0
	12 000	16 000	0	20	20	0	33 000	23 000	0	760 000			26.8	25.9	
p-fluorophenylalanine	34	134	62	260	698	214	25	4.2	3.5				8.3	3.7	3.2
	24	26	125	100	248	20	13.5	2.9	14.5				6.7	2.8	6.9
β-phenylserine	36	84	228	430	495	297	18	3.7	9.5				7.4	3.4	5.8
	38	140	460	85	200	23	23	22.5	46				8.1	8.0	9.9
β-thien-2-ylalanine	45	122	114	187	264	256	46	10	5.3				9.8	5.9	4.3
	67	130	84	87	248	9.2	41	15	21				9.6	7.0	7.8
2-amino-4-methylhex-4-enoic acid	143	720	510	160	271	165	170	5.6	38				13.2	4.4	9.4
	400	85	0	34	224	0	625	11					16.6	6.2	
mimisone	5 500	145	137	156	386	123	6 800	8	13.5				32.7	5.4	6.7
	0	0	0	0	0	0	0	0	0						

N-benzyl-L-phenylalanine		85	157	205	131	128	48	122		26	53				12.4	8.4	10.2	
		1 500	6 100	0	15	12.5	0	5 400		11 000					22.1	24		
N-benzyl-D-phenylalanine		1 000	8 300	462	130	278	92	1 500		636	62				18.8	16.6	10.6	
		30 000	26 000	0	50	7.2	0	27 000		97 000	0				26.3	29.6		
ochratoxin A		875	2 000	217	340	347	97	500		125	27				16.0	12.4	8.5	
		0	0	0	0	0	0	0		0	0							

[a] Concentration of phenylalanine equals concentration of analogue. [b] With consideration of in vivo concentrations.

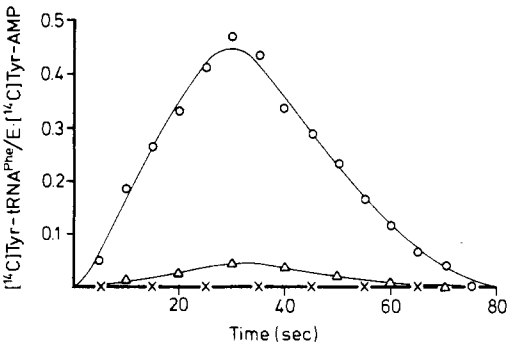


FIGURE 1: Transient formation of ^{14}C-labeled aa-tRNA^{Phe}-C-C-A during rejection of phenylalanine analogues by the phenylalanyl-tRNA synthetases from yeast and turkey liver at pH 7.65 and 0 °C. Enzyme–^{14}C-labeled aa-AMP (2.8 μM) was formed in situ in the presence of inorganic pyrophosphatase. Transfer of [^{14}C]tyrosine to tRNA^{Phe} (7 μM) by yeast (O) and tukey liver (×) phenylalanyl-tRNA synthetase. Transfer of *N*-benzyl-L-[^{14}C]phenylalanine to tRNA^{Phe} by the turkey liver phenylalanyl-tRNA synthease (Δ). Aliquots were spotted onto paper filter disks and immediately quenched with trichloroacetic acid.

of [^{14}C]phenylalanine that can be incorporated with a back-titration (see Experimental Procedures). The noncognate natural amino acids (tyrosine, leucine, methionine) are only detectable as aminoacyl-tRNA^{Phe}-C-C-A(3′NH$_2$), not as aminoacyl-tRNA^{Phe}-C-C-A in the *E. coli* and yeast systems (Table II, A and B). It is significant that in the turkey liver system noncognate natural amino acids are not transferred to tRNA^{Phe}-C-C-A(3′NH$_2$), but *p*-fluorophenylalanine, β-phenylserine, and β-thien-2-ylalanine could be isolated as aminoacyl-tRNA. These analogues were in fact substrates for all phenylalanyl-tRNA synthetases tested. *N*-Benzyl-L-phenylalanine was isolated as an aminoacyl-tRNA only in the case of *E. coli* enzyme and 2-amino-4-methylhex-4-enoic acid only in the case of the yeast enzyme. Results from the back-titration experiment where only low transfer of the analogue to the tRNA was indicated were confirmed by product analysis (see Experimental Procedures).

Some of the analogues that are readily activated are not at all transferable to tRNA^{Phe}, as determined by the method of back-titration. No transfer is generally seen for the larger analogues like mimosine and ochratoxin A.

Transient Formation of [^{14}C]*Aminoacyl-tRNA*^{Phe}*-C-C-A with Noncognate Amino Acids.* Enzyme–aminoacyladenylate complexes, E·aa-AMP, using tyrosine or leucine were prepared in situ with the enzymes from yeast and turkey liver. Under the described conditions, [^{14}C]tyrosine is transiently transferred to tRNA^{Phe} by the yeast phenylalanyl-tRNA synthetase upon mixing the enzyme–[^{14}C]tyrosyladenylate complex with the tRNA^{Phe}-C-C-A (Figure 1). This indicates that transfer of the amino acid to the tRNA^{Phe}-C-C-A(3′NH$_2$) used in quantitating aminoacylation is not an artifact. No such transfer can be detected for the turkey liver enzyme. Similar results were obtained for leucine. Figure 1 also indicates that *N*-benzyl-L-phenylalanyl-tRNA^{Phe}-C-C-A is transiently formed to the extent of about 3% with the turkey liver enzyme when the E·aa-AMP complex is mixed with tRNA. No transient formation of misacylated tRNA^{Phe}-C-C-A was observed with mimosine or ochratoxin A for all systems.

The addition of unfractionated tRNA and yeast aminoacyl-tRNA synthetases to trap any, e.g., [^{14}C]Tyr-AMP expelled into solution did not result in aminoacyl-tRNA in the yeast system and no acid-precipitable counts in the turkey liver system. However, in both cases no retention of enzyme aminoacyladenylate by nitrocellulose filters was observed. Since

Table II: Further Substrate Properties of Tested Amino Acids

	(A) % aminoacylation of $tRNA^{Phe}$-C-C-A(3′NH_2)[a]			(B) % aminoacylation of $tRNA^{Phe}$ [b]			(C) [^{14}C]AMP production [k_{cat} (min^{-1})][c]			(D) k_{cat}[$tRNA^{Phe}$-C-C-A(3′NH_2)]/k_{cat}(AMP production)[d]		
	E.c.	*S.c.*	t.l.	*E.c.*	*S.c.*	t.l.	*E.c.*	*S.c.*	t.l.	*E.c.*	*S.c.*	t.l.
phenylalanine (Phe)	100	100	100	100	100	100	1.3	8.4	3.8	81	30	8.2
tyrosine (Tyr)	77	88	0	0	0	0	0.54	6.8	0.3	9.7	6.2	
leucine (Leu)	8	68	0	0	0	0	0.07	0.64	2.9	14	1.6	
methionine (Met)	17	48	0	0	0	0	0.11	2	3.6	13	0.7	
p-fluorophenylalanine	93	97	78	45	62	57	1.53	6.2	1.1	62	38	16.5
β-phenylserine	94	96	55	85	68	40	0.6	4	2.1	125	46	6.6
β-thien-2-ylalanine	90	80	69	6	17	1	1.4	142	3.2	52	1.6	2.9
2-amino-4-methylhex-4-enoic acid	86	82	0	0	8	0	0.24	116	0	78	1.8	
mimosine	0	0	0	0	0	0	0	0.6	0			
N-benzyl-L-phenylalanine	64	50	0	7	<1	0	0.14	2.6	1.4	35	0.6	
N-benzyl-D-phenylalanine	26	20	0	0	0	0	0.04	0.04	0	6.5		
ochratoxin A	0	0	0	0	0	0	0	1.2	0			

[a] Amount of $tRNA^{Phe}$-C-C-A(3′NH_2) aminoacylated in 60 min with 1 mM amino acid. [b] Amount of $tRNA^{Phe}$-C-C-A aminoacylated in 5 min with 1 mM amino acid. [c] Turnover number of nonstoichiometric AMP production with 1 mM amino acid and $tRNA^{Phe}$-C-C-A. [d] Relation of turnover numbers of aminoacylation of $tRNA^{Phe}$-C-C-A(3′NH_2) and nonstoichiometry AMP production at 1 mM amino acid.

Table III: $tRNA^{Phe}$-C-C-N-Dependent [^{14}C]AMP Formation via Phenylalanyl-tRNA Synthetase from Turkey Liver and Bakers' Yeast at 1 mM Amino Acid

		k_{cat} (min^{-1})			
enzyme	source	Phe	Leu	Met	Tyr
phenylalanyl-tRNA synthetase	bakers' yeast	<0.05	<0.05	<0.05	<0.05
	turkey liver	0.70	0.75	1.35	<0.1
E·$tRNA^{Phe}$-C-C	bakers' yeast	<0.1	<0.05	<0.05	<0.05
	turkey liver	0.7	0.80	1.60	<0.1
E·$tRNA^{Phe}$-C-C-3′dA	bakers' yeast	<0.1	<0.1	<0.1	<0.1
	turkey liver	0.62	0.64	1.13	<0.1
E·$tRNA^{Phe}$-C-C-A	bakers' yeast	8.4	0.64	2.03	6.80
	turkey liver	3.8	2.93	3.62	0.31
E·$tRNA^{Phe}$-C-C-2′dA	bakers' yeast	<0.05	<0.05	<0.05	<0.05
	turkey liver	0.39	2.50	3.57	0.27
E·$tRNA^{Phe}$-C-C-A(3′NH_2)	bakers' yeast	0.89	0.1	0.25	0.72
	turkey liver	1.9	2.0	2.6	0.18

in the absence of enzyme the aminoacyladenylates have a half-life of 1–2 min, the noncognate aminoacyladenylates appear to be enzymatically hydrolyzed, rather than released into solution. A nonenzymic reaction of the free aminoacyladenylate with the 3′-amino group to account for aminoacylation of $tRNA^{Phe}$-C-C-A(3′NH_2) as suggested by Fersht & Dingwall (1979c) can therefore, at least in the Tris-buffer system, be excluded.

AMP Production in the Presence of $tRNA^{Phe}$-C-C-A. Phenylalanyl-tRNA synthetases hydrolyze ATP to AMP in the presence of noncognate amino acids that are activated by the enzymes. Amino acids that are not activated by the enzymes (e.g., glycine and lysine) do not result in AMP production. The formation of AMP during the charging reaction in nonstoichiometric amounts is an indication that an aminoacylation with a subsequent hydrolysis is occurring. The turnover number for the AMP production with 1 mM amino acid (Table II, C) can be compared to the turnover number for aminoacylation of $tRNA^{Phe}$-C-C-A(3′NH_2) at the same concentration. The quotient (Table II, D) is a measure of the proofreading capacity of a synthetase by indicating the number of aminoacylations per proofreading step. This ratio of the synthetic to the subsequent hydrolytic step is generally higher for phenylalanine than for all other analogues and indicates further enhancement of accuracy. The turnover numbers of the nonstoichiometric AMP production with phenylalanine are higher than with all other analogues; the relevant figure is, however, the ratio of turnover numbers for aminoacylation vs. nonstoichiometric AMP production. The values for the quotient presented in Table II, D are consistent with the stoichiometry of Phe-$tRNA^{Phe}$-C-C-A formation: (*E. coli* enzyme) 0.99, (yeast enzyme) 0.96, and (turkey liver enzyme) 0.92 nmol of Phe-$tRNA^{Phe}$/nmol of ATP.

Effect of Modification of tRNA 3′ Terminus on AMP Production. The influence of the 3′ terminus of $tRNA^{Phe}$ on AMP production is determined by using 3′-modified $tRNAs^{Phe}$ (Table III). Using an enzyme complex containing $tRNA^{Phe}$ lacking the 3′-terminal adenosine (E·$tRNA^{Phe}$-C-C) or free enzyme, it was observed that the misactivated intermediates have considerable stability in the presence of enzyme from bakers' yeast but are unstable in the presence of enzyme from turkey liver. In the case of the free enzyme, RNase A was employed as a control in order to exclude any AMP production due to traces of tRNA impurities in the enzyme preparation but was found to have no influence. With the two complexes enzyme–$tRNA^{Phe}$-C-C and enzyme–$tRNA^{Phe}$-C-C-3′dA or free enzyme, similar rates of AMP formation were observed. The modified tRNA containing a 3′-deoxyadenosine moiety cannot be misaminoacylated by other natural amino acids besides phenylalanine in the case of the turkey liver enzyme but is a substrate for the yeast enzyme. With this modified $tRNA^{Phe}$, proofreading in the yeast system, indicated by nonstoichiometric AMP formation, does not occur owing to the absence of the 3′-hydroxyl group that is essential for this process (Cramer et al., 1979). Enhanced nonstoichiometric AMP formation as a result of a proofreading process takes place with the enzyme–$tRNA^{Phe}$-C-C-A complex with enzymes from both organisms. The contribution to accuracy is seen, when this value is considered in relation to the turnover number of aminoacylation of $tRNA^{Phe}$-C-C-A(3′NH_2) (Table II, D).

The enzyme–$tRNA^{Phe}$-C-C-2′dA complex is neither a substrate for phenylalanylation in the yeast or turkey liver systems nor results in proofreading with the yeast enzyme. However, a very efficient hydrolysis of noncognate amino acid adenylates takes place with the turkey liver enzyme.

Nonstoichiometric ATP hydrolysis with respect to aminoacylation of $tRNA^{Phe}$-C-C-A(3′NH_2) is of interest. It would suggest that a small amount of deacylation must therefore occur from the 2′-acceptor OH prior to transacylation to the 3′-NH_2. After a number of enzyme cycles, the buildup of the transacylation product with its stable 3′-amide will dominate, and ATP hydrolysis ceases. No enzymatic hydrolysis of the 3′-amide bond could be seen with any enzyme tested. $tRNA^{Phe}$-C-C-A(3′NH_2) acts as an acceptor and appears to have a small proofreading capability. Similar results were obtained with the yeast valyl-tRNA synthetase by Igloi et al. (1977).

No AMP production with mimosine and ochratoxin A was observed with $tRNA^{Phe}$-C-C-2′dA and the yeast enzyme; additionally, no transient formation of misacylated $tRNA^{Phe}$-C-C-A was observed. This indicates that a rapid proofreading hydrolysis follows the transfer of the amino acid to the 2′-OH of the tRNA to explain the measurable AMP production in the presence of $tRNA^{Phe}$-C-C-A.

The turkey liver enzyme transfers phenylalanine, no other natural amino acid, but some analogues like β-thien-2-ylalanine and *N*-benzyl-L-phenylalanine to $tRNA^{Phe}$-C-C-3′dA. The AMP production with the nonaccepting $tRNA^{Phe}$-C-C-2′dA and the turkey liver enzyme is 10% of the value observed in the presence of $tRNA^{Phe}$-C-C-A for phenylalanine and these analogues. The difference from values measured in the presence of natural amino acids like leucine and methionine suggests a different mode of discrimination.

Discussion

Phenylalanyl-tRNA synthetases from different sources will promote ATP/PP_i pyrophosphate exchange with a wide variety of structurally dissimilar amino acids. Neither a closed phenyl ring nor an unsubstituted α-NH_2 group is essential. Additionally, a larger ring system can be tolerated. The lack of specificity for phenylalanyl-tRNA synthetases was noted earlier in the case of the yeast enzyme (Igloi et al., 1978; Igloi & Cramer, 1978).

The difference of the kinetic parameters upon binding of $tRNA^{Phe}$ compared to activation indicates an improvement in amino acid discrimination and a probable narrowing of the binding pocket for the amino acid. This may be mediated by the enzyme via a conformational change triggered by the 3′-adenosine $tRNA^{Phe}$ (von der Haar & Gaertner, 1975; Fasiolo et al., 1981). A similar change for the kinetic parameters was noted for the *E. coli* enzyme by Santi et al. (1971).

Only minor differences are detectable between the enzymes in the hydrophobic pocket, with its stringent steric requirements for the phenyl ring type moiety. This is substantiated by the data of the pyrophosphate exchange (Table I), indicating that the turkey liver enzyme has more tolerance with respect to the inclusion of the *p*-hydroxyl group of tyrosine into the hydrophobic microenvironment and the energetically unfavorable removal of one or more water molecules from the hydration sphere surrounding the aromatic hydroxyl.

The different discrimination mechanism by the turkey liver enzyme is further demonstrated by the fact that natural amino acids other than phenylalanine are not transferred to $tRNA^{Phe}$. Additional comparison for the effect of the complexed tRNA is only possible with the data of the AMP/PP_i independent hydrolysis, given in Table II. The low k_{cat} of AMP production indicates the mutually improved selection specificity in the presence of tRNA.

p-Fluorophenylalanine and β-phenylserine can be isolated as a stable aminoacyl-tRNA without significant species-specific differences. In the case of β-thien-2-ylalanine, the reduction of the ring size is partially compensated by dispersion forces, which are reported to be 2.5× greater between –S– and $-CH_2-$ than between $-CH_2-$ and $-CH_2-$ and thus increase the binding energy for substitution of –S– for $-CH_2-$ by 13 kJ/mol (Fersht & Dingwall, 1979b).

The planar system of 2-amino-4-methylhex-4-enoic acid, tolerated by the yeast enzyme, appears very unfavorable for the *E. coli* enzyme. As with the natural amino acids, the turkey liver enzyme gives no aminoacylation of $tRNA^{Phe}$-C-C-A(3′NH_2) or $tRNA^{Phe}$-C-C-A with this analogue.

As indicated by Santi & Webster (1976), *E. coli* phenylalanyl-tRNA synthetase possesses, in addition to the phenyl-binding pocket, a second hydrophobic area near the α-NH_2 group. A similar weak hydrophobic area is also indicated by the present data; however, access to it is restricted by complexation with tRNA. So *N*-benzyl-L-phenylalanine containing a hydrophobic α-amino substituent is activated by all tested enzymes; however, transfer to the tRNA is only detected for the *E. coli* enzyme and to a very small extent for the yeast enzyme. No aminoacylation of $tRNA^{Phe}$-C-C-A(3′NH_2) could be measured for the turkey liver enzyme. This aspect of species-dependent differences and its implication of pharmacological application have been discussed by von der Haar et al. (1981).

From the data it appears that the initial recognition of the amino acid by the free enzyme is most accurate in the case of *E. coli*. This difference in accuracy and its subsequent effect on aminoacylation is only valid in a comparison with the yeast enzyme. The turkey liver enzyme on the other hand appears to have developed a different approach in order to obtain a high fidelity of aminoacylation. In agreement with the data from the cysteinyl-tRNA synthetase of *E. coli* (Fersht & Dingwall, 1979b), a lower proofreading capacity can be expected for the *E. coli* enzyme, owing to high initial amino acid recognition with an error rate that is close to 10^{-6} with noncognate natural amino acids (Table I).

In this respect, the results of the AMP/PP_i independent hydrolysis of aminoacyl-tRNA are of special importance. With the quotient of the turnover numbers of aminoacylation of $tRNA^{Phe}$-C-C-A(3′NH_2) and nonstoichiometric AMP production, it can be deduced that accurate initial recognition processes correlate with less elaborate hydrolytic capacity.

The ratio of the primary synthetic step and the subsequent hydrolytic step occurring during aminoacylation is significantly smaller in the case of noncognate natural amino acids than in the case of phenylalanine for the *E. coli* and yeast enzyme and indicates a further enhancement of accuracy after initial recognition of the amino acid by the enzyme.

Since we have shown that the transfer of amino acid to $tRNA^{Phe}$-C-C-A(3′NH_2) is applicable to the normal catalytic cycle, it can be concluded from the data that the route of proofreading differs for the enzymes. With the turkey liver enzyme, natural amino acids promote ATP hydrolysis without being covalently bound to the tRNA. This is clearly a case of pretransfer proofreading in the terminology of Jakubowski & Fersht (1981). AMP production in the presence of modified $tRNAs^{Phe}$ implicates the 3′-OH group of the terminal adenosine in this reaction. From the significant ATP hydrolysis, occurring nonstoichiometrically with respect to aminoacylation

of $tRNA^{Phe}$-C-C-A(3′NH_2), and the exclusion of dissociation of noncognate aminoacyladenylates from the enzyme, one can conclude that some chemical proofreading occurs even in the case where an amino group exists at the adjacent 3′-OH position.

While natural amino acids other than phenylalanine are not transferred to $tRNA^{Phe}$-C-C-A(3′NH_2) or -3′dA, other analogues like β-thien-2-ylalanine are isolatable as aminoacyl-$tRNA^{Phe}$. This together with the AMP production indicates the presence of a hydrolytic step after transfer of the amino acid to the tRNA. This is substantiated by the fact that transient formation of *N*-benzyl-L-phenylalanyl-$tRNA^{Phe}$-C-C-A can be detected without observation of aminoacyl-$tRNA^{Phe}$-C-C-A(3′NH_2). In this case, hydrolysis on the enzyme surface appears to proceed faster than transacylation. Other analogues (2-amino-4-methylhex-4-enoic acid, mimosine, *N*-benzyl-D-phenylalanine) are not activated even in the presence of tRNA. Thus the pathways available to proofread errors of amino acid misactivation by the turkey liver enzyme are dependent on the structure of the amino acid. The posttransfer proofreading pathway is observed with the other two enzymes. Preferential posttransfer hydrolysis appears to occur in the yeast enzyme with less bulky substituents, explaining their relatively low amount of isolatable aminoacyl-tRNA. This rapid deacylation of tRNA aminoacylated with smaller incorrect substrates resembles the deacylation of Val-$tRNA^{Ile}$ or α-aminobutyryl-$tRNA^{Val}$ (Igloi et al., 1977; Fersht & Dingwall, 1979c). An interesting observation is the fact that the hydrolysis of β-phenylserinyl-$tRNA^{Phe}$ is less than that for phenylalanine (125:81 and 46:30, as expressed in the ratio of synthetic to subsequent hydrolytic step). Binding of the analogue here can result in steric exclusion of a water molecule by the OH group of β-phenylserine. A different binding of the analogue and positioning of the β-OH group by the turkey liver enzyme are indicated in the lower quotient for β-phenylserine (6.6:8.2).

Considering the capability of the turkey liver enzyme to use pretransfer proofreading for noncognate natural amino acids and posttransfer proofreading for synthetic analogues, it can be suggested that there is probably not a fundamental mechanistic difference between the pretransfer and posttransfer pathway. The interplay between the active site of the enzyme, the 3′-terminal adenosine, and the aminoacyladenylate may give an intermediary complex that potentially can dissipate its energy into either pathway dependent on the structure of the amino acid. Donor and acceptor interactions within the active site, perhaps via a carboxylate, the 3′-OH group and a histidine residue, may result in a correct orientation and necessary polarization, common to both subsequent reactions. For example, in the case of tyrosine, either the amino acid is transiently transferred to the 2′-OH group and proofread via the influence of the 3′-OH group (*E. coli*, *S. cerevisiae*), or the aminoacyl moiety of the aminoacyladenylate is positioned for hydrolysis before transfer (turkey liver).

Thus the different enzymes appear to use different strategies such that the overall cost of proofreading, as calculated by Savageau & Freter (1979), will decrease until the energy by an additional decrement in proofreading is equal to the increment in energy waste resulting from a concomitant increase in net error. The valyl-tRNA synthetases of *E. coli*, *Bacillus stearothermophilus*, yeast, and *Lupinus luteus*, for example, all follow primarily the posttransfer pathway (Fersht & Kaethner, 1976; Igloi et al., 1978; Fersht & Dingwall, 1979a; Jakubowski & Fersht, 1981).

In summary, the phenylalanyl-tRNA synthetases from *E. coli*, yeast, *N. crassa*, and turkey liver that have similar molecular weight and an $\alpha_2\beta_2$ subunit structure (Joachimiak & Barciszewski, 1980; Gabius & Cramer, 1982) show species-dependent differences observable when phenylalanine analogues are used. Initial recognition and hydrolytic capacity are coupled, which result in economic ATP consumption. A simple extrapolation from prokaryotic to eukaryotic systems is not applicable. Additionally, within the eukaryotes there are differences in the ratio between primary recognition vs. proofreading and also in the mechanism of proofreading (pretransfer and posttransfer).

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Registry No. Phe, 63-91-2; Tyr, 60-18-4; Leu, 61-90-5; Met, 63-68-3; *p*-fluoro-DL-phenylalanine, 51-65-0; DL-β-phenylserine, 69-96-5; β-thien-2-ylalanine, 139-86-6; 2-amino-4-methylhex-4-enoic acid, 17781-05-4; mimosine, 500-44-7; *N*-benzyl-L-phenylalanine, 19461-04-2; *N*-benzyl-D-phenylalanine, 85114-36-9; ochratoxin A, 303-47-9; phenylalanyl-tRNA synthetase, 9055-66-7; aminoacyl-tRNA synthetase, 9028-02-8.

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Identification of Tyrosine Residues That Are Susceptible to Lactoperoxidase-Catalyzed Iodination on the Surface of *Escherichia coli* 30S Ribosomal Subunit†

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ABSTRACT: The detailed surface topography of the *Escherichia coli* 30S ribosomal subunit has been investigated, with iodination catalyzed by immobilized lactoperoxidase as the surface probe. Under mild conditions, only proteins S3, S7, S9, S18, and S21 were iodinated to a significant and reproducible extent. These proteins were isolated from the iodinated subunits, and in each case, the individual tyrosine residues that had reacted were identified by standard protein sequencing techniques. The targets of iodination that could be positively established were as follows: in protein S3 (232 amino acids), the tyrosines at positions 167 and 192; in S7 (153 amino acids), tyrosines 84 and 152; in S9 (128 amino acids), tyrosine 89; in S18 (74 amino acids), tyrosine 3 (tentative); in S21 (70 amino acids), tyrosines 37 and 70. The results represent part of a broader program to investigate ribosomal topography at the amino acid–nucleotide level.

Chemical modification of ribosomal proteins in situ is an approach that has been used in the past by many research groups in order to probe the surface topography of the *Escherichia coli* ribosome. In the earlier experiments [see, e.g., Benkov & Delihas (1974) and Brimacombe et al. (1976) for reviews] the objective was to determine which of the ribosomal proteins were buried in the structure and which of them were accessible on the surface, but as the data began to accumulate, it soon became clear that most if not all the proteins could be modified to a greater or lesser extent by one reagent or another. Interpretation of the data was very difficult, since each reagent showed a different spectrum of reactivity toward the individual proteins, and it was also impossible to determine to what extent some of the smaller reagents were able to penetrate into the structure. Errors and ambiguities in the identification of the modified proteins by gel electrophoresis undoubtedly added to the confusion. As a result, the chemical-modification approach has tended not to be favored in recent years.

However, chemical modification can yield useful and important data, provided that three preconditions are met. The

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