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Evolutionary Aspects of Accuracy of Phenylalanyl-tRNA Synthetase. Accuracy of the Cytoplasmic and Chloroplastic Enzymes of a Higher Plant (*Phaseolus vulgaris*)†

Reinhard Rauhut, Hans-Joachim Gabius, and Friedrich Cramer*
Max-Planck-Institut für Experimentelle Medizin, Abteilung Chemie, D-3400 Göttingen, Federal Republic of Germany
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ABSTRACT: The phenylalanyl-tRNA synthetases from cytoplasm and chloroplasts of bean (*Phaseolus vulgaris*) leaves employ different strategies with respect to accuracy. The chloroplastic enzyme that is coded for by the nuclear genome follows the pathway of posttransfer proofreading, also characteristic for enzymes from eubacteria and cytoplasm and mitochondria of lower eukaryotic organisms. In contrast, the cytoplasmic enzyme uses pretransfer proofreading in the case of noncognate natural amino acids, characteristic for higher eukaryotic organisms and archaebacteria. Dependent on the nature of the noncognate amino acid, pretransfer proofreading in this case occurs without tRNA stimulation or with tRNA stimulated with no or little effect of the nonaccepting 3′-OH group of the terminal adenosine. The fundamental mechanistic difference in proofreading between the heterotopic intracellular isoenzymes of the plant cell supports the idea of the origin of the chloroplastic gene by gene transfer from a eubacterial endosymbiont to the nucleus. Origin by duplication of the nuclear gene, as indicated for mitochondrial phenylalanyl-tRNA synthetases [Gabius, H.-J., Engelhardt, R., Schroeder, F. R., & Cramer, F. (1983) *Biochemistry 22*, 5306–5315], appears unlikely. Further analyses of the ATP/PP_i pyrophosphate exchange and aminoacylation of $tRNA^{Phe}$-C-C-A(3′NH_2), using 11 phenylalanine analogues, reveal intraspecies and interspecies variability of the architecture of the amino acid binding part within the active site.

It is well established that the correct aminoacylation of the cognate tRNA with the proper amino acid by aminoacyl-tRNA synthetases strongly contributes to the overall fidelity of protein synthesis (Cramer et al., 1979; Yarus, 1979). Recently we have presented evidence in the case of the phenylalanyl-tRNA synthetases that the strategies to achieve the high level of fidelity in this process differ among various organisms at different branches of the evolutionary tree (Gabius et al., 1983a,b). Between the various organisms the contribution of primary recognition of the amino acid and the successive corrective step, proofreading, to fidelity differs markedly. Within the eukaryotes even the proofreading mechanism, whereby misactivated intermediates are hydro-

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lyzed before or after transfer to tRNA, reveals species-specific differences. In this respect, aminoacyl-tRNA synthetases from green plants and their different intracellular compartments have not been studied thoroughly due to the inherent problems of enzyme purification, because plant extracts are very rich in potentially harmful substances from their secondary metabolism as a variety of phenolic compounds, tannins, and pigments (Van Sumere et al., 1975).

In this paper we thus extend our evolutionary study of phenylalanyl-tRNA synthetases to the cytoplasmic and chloroplastic enzymes from a green higher plant (bean, *Phaseolus vulgaris*), in order to establish a more comprehensive picture for the strategies to achieve the necessary fidelity in the various kingdoms of organisms. This comparison between these heterotopic intracellular isoenzymes with their crucial role in cytoplasmic and chloroplastic protein biosynthesis, respectively, is also of special interest for the question of chloroplast origin. Furthermore, the analysis of substrate analogues for the amino acid provides an approach to uncover subtle differences in the topography of the active site of related enzymes and aids in assessing phylogenetic relationships.

Experimental Procedures

Materials. Bean (*Phaseolus vulgaris*) seeds from Van Waveren (Goettingen, FRG) germinated in optimal green house conditions with 12 h light/12 h dark periods at 22–24 °C for about 10 days. Cytoplasmic phenylalanyl-tRNA synthetase (EC 6.1.1.20) was purified from leaves 2800-fold to homogeneity according to accepted criteria (Rauhut et al., 1984). Chloroplastic phenylalanyl-tRNA synthetase was purified under optimal protective conditions over 4900-fold to homogeneity, exhibiting a unique dimeric subunit structure of $\alpha\beta$ compared to any characterized phenylalanyl-tRNA synthetase and a drastic size reduction of 40% compared to the cytoplasmic enzyme (R. Rauhut et al., unpublished results). Activity was routinely checked by active site titration (Fersht et al., 1975). Absence of any contaminating enzymatic activity was established by using different ^{14}C-labeled amino acids and an unfractionated tRNA mixture as acceptor (Igloi et al., 1979). tRNA^{Phe}-C-C and tRNA^{Phe}-C-C-A were isolated by the procedure of Schneider et al. (1972) from unfractionated bakers' yeast tRNA and *Escherichia coli* tRNA. Incorporation of 3′-deoxy-, 2′-deoxy-, and 3′-deoxy-3′-aminoadenosine 5′-phosphates into tRNA^{Phe}-C-C using tRNA nucleotidyl transferase (EC 2.7.7.25), kindly provided by Dr. H. Sternbach, was performed as described (Sprinzl & Sternbach, 1979). tRNA^{Phe} from bean cytoplasm and bean chloroplasts was isolated essentially as described (Everett & Madison, 1976). Sources for amino acids and radiochemicals have been given in detail in previous papers (Gabius et al., 1983a,b). To rigorously exclude any contamination of commercial tyrosine by phenylalanine, tyrosine was successively recrystallized in water as outlined by Lin et al. (1983). Contamination of any amino acid by phenylalanine was excluded by the absence of reduction of aminoacylation with [^{14}C]phenylalanine for 250-fold excess of the nonradioactive amino acid under consideration in the assay (isotope dilution). Other chemicals were commercially available analytical grade.

Kinetic Procedures. ATP/PP$_i$ pyrophosphate exchange, aminoacylation of unfractionated tRNA and tRNA^{Phe} with radioactive amino acid, aminoacylation of tRNA^{Phe}-C-C-A and tRNA^{Phe}-C-C-A(3′NH$_2$) in the course of back-titration to quantitate the transfer of nonradioactive amino acid to tRNA^{Phe}, and AMP production in the presence of tRNA^{Phe}-C-C-A and tRNA^{Phe}s modified at the terminal adenosine were measured by conventional procedures without any change in the conditions (Gabius et al., 1983a,b). Generally, tRNA^{Phe} from yeast was the substrate for the cytoplasmic enzyme, and tRNA^{Phe} from *E. coli* was the substrate for the chloroplastic enzyme for the data summarized in Tables I–III. Controls using the homologous system are outlined under Results.

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, 1977)

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

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 (1977):

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

Results

Activation of Phenylalanine Analogues. It has recently been found that phenylalanyl-tRNA synthetases from different sources (*E. coli*, yeast, *N. crassa*, turkey liver, mitochondria of yeast and hen liver) activate a number of phenylalanine analogues (Gabius et al., 1983a,b). By use of enzyme preparations and amino acids, carefully checked for purity to avoid misinterpretations, the kinetic parameters of activation of the analogues, as monitored by the ATP/PP$_i$ pyrophosphate exchange in the absence of tRNA^{Phe}, give a first indication of specificity for the recognition of amino acid analogues by the different plant phenylalanyl-tRNA synthetases. Both plant enzymes, which have so far not been the subject of extensive amino acid analogue study, promote ATP/PP$_i$ pyrophosphate exchange with all phenylalanine analogues, including the natural noncognate amino acids tyrosine, leucine, and methionine (Table I). The kinetic constants generally show an increased K_m and a decreased k_{cat} compared to phenylalanine in both cases. Exceptions for the cytoplasmic enzyme in this respect consist of 2-amino-4-methylhex-4-enoic acid and *N*-benzyl-D-phenylalanine, where the corresponding k_{cat} exceeds the value measured with phenylalanine. This increase in k_{cat} and not in K_m may reflect a conversion to a more active conformation, indicating structural flexibility within the active site. For the chloroplastic enzyme different analogues reveal deviations from this general pattern. An increased value of k_{cat} is measured for methionine, and a decreased value of K_m is measured for *p*-fluorophenylalanine and β-thien-2-ylalanine. The kinetic parameters allow one to calculate the relative specificity against noncognate amino acids, expressed as V_{Phe}/V_{Xxx} (see Experimental Procedures). These data demonstrate that only a limited structural relationship to phenylalanine is necessary to allow binding and misactivation in both cases. As a first measure for the relative specificities of the two enzymes, the discrimination of the cytoplasmic enzyme against noncognate amino acids at this stage significantly surpasses the corresponding ability of the chloroplastic enzyme. This extent of differentiation between cognate and noncognate amino acids is clearly not sufficient to bring about the required specificity in both cases. In comparison to other enzymes, both sets of data do not clearly fit in any other set and demonstrate structural differences in the amino acid binding site for the enzyme from different organisms.

Table I: Substrate Properties of the Tested Amino Acids for Phenylalanyl-tRNA Synthetases from Bean[a]

amino acid	K_m (μM)		k_{cat} (min^{-1})		V_{Phe}/V_{Xxx}[b]		$\Delta\Delta G_B$ (kJ/mol)	
	cyto	chloro	cyto	chloro	cyto	chloro	cyto	chloro
phenylalanine (Phe)	40	43	180	67				
	7	32	34	6.3				
tyrosine (Tyr)	1000	1990	51	55	90	56	11.6	10.4
	c	295	c	0.2	c	363	c	15.2
leucine (Leu)	900	500	20	60	200	13	13.5	6.6
	c	c	c	c	c	c	c	c
methionine (Met)	3300	1060	39	110	450	15	15.7	7
	c	1735	c	0.3	c	1220	c	18.3
p-fluorophenylalanine	63	9	76	12.5	4	1	3.4	0.3
	235	125	80	4	14	6	6.8	4.7
β-phenylserine	190	75	90	24	10	5	5.8	4.1
	670	92	10	4.7	324	4	14.8	3.5
β-thien-2-ylalanine	124	18	98	19	9	2	5.5	1
	606	465	46	10.6	67	9	10.8	5.5
2-amino-4-methylhex-4-enoic acid	2150	750	252	42	38	28	9.3	8.6
	3570	6000	51	32	350	37	15.1	9.3
mimosine	1270	500	35	68	150	12	12.9	6.3
	c	c	c	c	c	c	c	c
N-benzyl-L-phenylalanine	750	370	22	52	150	11	12.9	6.2
	1400	375	2	0.4	3470	185	21.0	13.5
N-benzyl-D-phenylalanine	6250	200	222	46	115	7	12.2	4.9
	c	c	c	c	c	c	c	c
ochratoxin A	980	5600	74	46	56	190	10.4	13.5
	450	940	0.3	0.9	6480	206	22.6	13.7

[a] The first line always gives the data for the pyrophosphate exchange; the second line, the data for aminoacylation of tRNA-C-C-A(3′NH_2). [b] Concentration of Phe equals that of the analogue. [c] Dash, not substrate.

Kinetic Data for Aminoacylation of tRNA^{Phe}-C-C-A-(3′NH_2) with Phenylalanine Analogues. With tRNA^{Phe}-C-C-A(3′NH_2), where the potentially labile ester bond of the aminoacyl-tRNA is replaced by a stabile amide link, the transfer of misactivated amino acid analogues to the 3′-group of the terminal adenosine can be quantified. The contribution of tRNA to the selection of noncognate amino acids can thus be determined. Certain amino acids that are activated, as measured by the ATP/PP_i pyrophosphate exchange, may not be substrates in aminoacylation of tRNA. The cytoplasmic enzyme does not transfer the natural noncognate amino acids, mimosine, and *N*-benzyl-D-phenylalanine to tRNA^{Phe}-C-C-A(3′NH_2). In contrast, the chloroplastic enzyme transfers the natural amino acids tyrosine and methionine to tRNA^{Phe}-C-C-A(3′NH_2). This constitutes a fundamental difference between these intracellular heterotopic isoenzymes.

Formation of the binary enzyme-tRNA complex enhances the specificity relative to the free enzyme for the other analogues transferred by both enzymes, as expressed in terms of V_{Phe}/V_{Xxx} (Table I). The increase in specificity appears relatively higher for the cytoplasmic enzyme than for its chloroplastic counterpart, adding another difference in their properties to the comparison. The calculated values for V_{Phe}/V_{Xxx} were experimental shown to be equivalent to the excess of analogue necessary to produce a 50% inhibition of phenylalanine aminoacylation. As noted before for the cytoplasmic enzyme, 2-amino-4-methylhex-4-enoic acid and also β-thien-2-ylalanine and *p*-fluorophenylalanine exhibit higher k_{cat} values than phenylalanine. Only the analogues 2-amino-4-methylhex-4-enoic acid and β-thien-2-ylalanine are transferred to tRNA^{Phe}-C-C-A(3′NH_2) with a higher k_{cat} than that of phenylalanine by the chloroplastic enzyme. The specificity of the cytoplasmic enzyme, as expressed in terms of V_{Phe}/V_{Xxx}, surpasses that for the chloroplastic enzyme, as noted before in ATP/PP_i pyrophosphate exchange. Especially the β-OH group of β-phenylserine appears unfavorable for the interaction with the cytoplasmic enzyme. The bulky substituent on the α-NH_2 group in ochratoxin A does not impair transfer in both cases.

Aminoacylation of tRNA^{Phe}-C-C-A(3′NH_2) and tRNA^{Phe}-C-C-A with Phenylalanine Analogues. To elucidate differences in the aminoacylation of tRNA^{Phe}-C-C-A(3′NH_2) and tRNA^{Phe}-C-C-A, indicative for proofreading at the level of misaminoacylated tRNA^{Phe}, the nonradioactive amino acids were preincubated with enzyme and tRNA. The amount of residual nonaminoacylated tRNA is determined via back-titration by the extent of subsequent [^{14}C]phenylalanine incorporation. Whereas *p*-fluorophenylalanine, β-phenylserine, β-thien-2-ylalanine, and 2-amino-4-methylhex-4-enoic acid are isolable as aminoacyl-tRNA^{Phe}-C-C-A, *N*-benzyl-L-phenylalanine and ochratoxin A are only detectable as aminoacyl-tRNA^{Phe}-C-C-A(3′NH_2) (Table II, A and B). This series of aminoacylation with analogues reveals only quantitative differences between the plant enzymes but some qualitative differences to all previously described patterns. The property of transfer of ochratoxin A to tRNA^{Phe}-C-C-A(3′NH_2) is only shared by an archaebacterial enzyme (Rauhut et al., 1985); transfer of 2-amino-4-methylhex-4-enoic acid to tRNA^{Phe}-C-C-A is only seen with the yeast cytoplasmic enzyme and mitochondrial enzymes from yeast and liver. In contrast to yeast, eubacterial, and archaebacterial enzymes, *N*-benzyl-D-phenylalanine is not transferred.

The noncognate natural amino acids are not detectable as aminoacyl-tRNA^{Phe}-C-C-A or aminoacyl-tRNA^{Phe}-C-C-A-(3′NH_2) for the cytoplasmic enzyme, as has been noted before only for liver enzymes and the archaebacterial enzyme. Attempts to show any transient formation of a noncognate aminoacyl-tRNA^{Phe}-C-C-A with enzyme–aminoacyl adenylate by using radioactive noncognate natural amino acids are unsuccessful. Furthermore, no aminoacylation of tRNA^{Phe}-C-C-3′dA, lacking the nonaccepting 3′-OH group, can be detected with noncognate natural amino acids. This modified tRNA is not a substrate only in the case of the enzymes from cytoplasm and mitochondria of hen liver that use a corrective step prior to transfer of the noncognate natural amino acid to the tRNA, pretransfer proofreading (Gabius et al., 1983a,b). These data suggest a corrective step without transfer also for the cytoplasmic plant enzyme.

Table II: Further Substrate Properties of the 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]	
	cyto	chloro	cyto	chloro	cyto	chloro	cyto	chloro
phenylalanine (Phe)	100	100	100	100	12	0.4	3	15.3
tyrosine (Tyr)	0	61	0	0	7.0	<0.1		
leucine (Leu)	0	0	0	0	0.2	0		
methionine (Met)	0	13	0	0	3.8	<0.1		
p-fluorophenylalanine	90	93	76	51	18	0.8	4	4.5
β-phenylserine	88	97	49	93	10.2	0.4	0.6	12.3
β-thien-2-ylalanine	96	93	49	61	46.7	0.5	0.6	14.1
2-amino-4-methylhex-4-enoic acid	83	88	66	19	15.9	0.4	1.6	11.6
mimosine	0	0	0	0	0.8	0		
N-benzyl-L-phenylalanine	17	69	0	0	2.1	0.2	0.4	1.8
N-benzyl-D-phenylalanine	0	0	0	0	0.2	0		
ochratoxin A	50	65	0	0	4.5	0.4	0.05	1.1

[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 nonstoichiometric AMP production at 1 mM amino acid.

Table III: tRNA^{Phe}-C-C-N Dependent [^{14}C]AMP Formation via Phenylalanyl-tRNA Synthetase from Bean Cytoplasm at 1 mM Amino Acid

	K_{cat} (min^{-1})				
enzyme	phenyl-alanine	tyrosine	methio-nine	β-thien-2-ylalanine	2-amino-4-methyl-hex-4-enoic acid
phenylalanyl-tRNA synthetase	1.1	6.0	3.9	1.5	9.7
E-tRNA^{Phe}-C-C	0.8	6.1	3.8	10.7	10.6
E-tRNA^{Phe}-C-C-3′dA	1.4	5.2	3.8	12.2	3.9
E-tRNA^{Phe}-C-C-A	12.0	7.0	3.8	46.7	15.9
E-tRNA^{Phe}-C-C-2′dA	1.7	6.8	4.0	12.5	8.6
E-tRNA^{Phe}-C-C-A(3′NH$_2$)	4.6	5.6	3.8	16.7	12.1

The transfer of tyrosine and methionine to tRNA^{Phe}-C-C-A(3′NH$_2$) by the chloroplastic enzyme resembles a corresponding ability of the eubacterial enzyme and the yeast cytoplasmic and mitochondrial enzymes. To exclude an influence of the source of tRNA^{Phe}-C-C-A in these reactions, both bean tRNA^{Phe}s were employed under the described conditions, yielding results that are similar to the yeast and *E. coli* tRNA within the limits of error. As a further control, these experiments, the ATP/PP$_i$ pyrophosphate exchange and the AMP production, are performed at the temperature of plant growth (22 °C) to exclude any qualitative change of mechanism with temperature change in this range. Only corresponding quantitative changes with temperature change can be detected (data not shown).

AMP Production in the Presence of tRNA^{Phe}-C-C-A. In the course of the aminoacylation reaction, ATP is hydrolyzed to AMP. Amino acids that are not activated by the enzyme (e.g., alanine and arginine) do not result in any AMP production. Under the described conditions, the nonstoichiometric generation of AMP is due to continuous hydrolysis of aminoacyl-tRNA or aminoacyl adenylate. It is therefore an appropriate indication that a corrective hydrolysis of the aminoacyl adenylate preceding aminoacylation or 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$_2$), where transfer is irreversible, at the same concentration. The quotient (Table II, D) with each amino acid is a measure of the proofreading capacity of an aminoacyl-tRNA synthetase. It indicates the number of aminoacylations, measured by formation of an amide bond to the 3′-NH$_2$ group of tRNA^{Phe}-C-C-A(3′NH$_2$), per corrective step. These values for the cytoplasmic enzyme are the lowest ratios of synthetic to subsequent hydrolytic step measured so far for phenylalanyl-tRNA synthetases. As some of the quotients are significantly lower than 1, this raises evidence that either deacylation from the 2′-acceptor OH group proceeds faster than transacylation to the 3′-NH$_2$ group or the noncognate aminoacyl adenylates are hydrolyzed before transfer. As noted earlier for the initial discrimination by recognition, the chloroplastic enzyme reveals a comparatively lower hydrolytic activity than the cytoplasmic enzyme.

Effect of Modification of tRNA 3′-Terminus on AMP Production. A series of experiments has been carried out to determine how modification of the terminal adenosine of the tRNA^{Phe}-C-C-A affects the AMP production with noncognate natural, a naturally occurring and a synthetic phenylalanine analogue (Table III). These experiments have only been meaningful for the cytoplasmic enzyme. The basal level of AMP production with tRNA^{Phe}-C-C-A is too low for the chloroplastic enzyme to allow accurate determination of differences with modified tRNAs^{Phe}. This resembles the properties of eubacterial enzymes.

It is apparent from the data for the cytoplasmic enzyme that tRNA exerts different effects on the AMP production, depending on the nature of the amino acid. Misactivated natural amino acids appear unstable with all complexes and even with the free enzyme. With leucine only insignificant ATP hydrolysis occurs in all cases with no detectable effect of modification of terminal adenosine of tRNA (not shown), comparable to the negligible AMP production, measured so far only with mitochondrial phenylalanyl-tRNA synthetases from yeast and hen liver.

Whereas the methionine-dependent AMP production is not influenced by the presence of the different types of tRNA^{Phe}, tRNA^{Phe}-C-C-A has a slight stimulatory effect on tyrosine-dependent AMP production. This slight stimulation seems to be influenced in different ways by the vicinal 2′- and 3′-OH

groups. Only the presence of the nonaccepting 3′-OH group that cannot be substituted by an NH_2 group assures the stimulatory effect of tRNA. A similar gradation between the stability of the activated amino acid 2-amino-4-methylhex-4-enoic acid is seen in the presence of $tRNA^{Phe}$-C-C-2′dA and $tRNA^{Phe}$-C-C-3′dA, where again the intermediate does not have considerable stability. Nonstoichiometric ATP hydrolysis with respect to aminoacylation of $tRNA^{Phe}$-C-C-A(3′NH_2) suggests involvement of deacylation from the 2′-acceptor OH group prior to transacylation to the 3′-NH_2. On the other hand, the AMP production with the synthetic analogue β-thien-2-ylalanine is strongly stimulated by tRNA. Even the presence of $tRNA^{Phe}$-C-C is sufficient to generate a 7-fold stimulatory effect. This considerably diminished stability of enzyme-bound noncognate aminoacyl adenylate in the presence of $tRNA^{Phe}$-C-C and $tRNA^{Phe}$s including modifications of the terminal adenosine indicates a further significant enhancement of hydrolysis of noncognate aminoacyl adenylate by tRNA in this special case. The data of Table III indicate that tRNA, when bound to the enzyme, can influence the hydrolysis of enzyme-bound noncognate aminoacyl adenylates in various ways, depending on the structure of the amino acid. No stimulatory effect, stimulation even in presence of $tRNA^{Phe}$-C-C lacking the terminal adenosine that is supposedly important for reactive site triggering in the yeast enzyme (von der Haar & Gaertner, 1975; Fasiolo et al., 1981), and slight stimulation by the 3′-OH group of the terminal adenosine of tRNA have so far not been described for a single enzyme.

Discussion

We have previously reported a relative lack of specificity in the initial recognition of phenylalanine analogues by phenylalanyl-tRNA synthetases. Upon binding of tRNA, amino acid discrimination is improved, as changes of the kinetic parameters indicate. This appears to be a general feature of eubacterial and eukaryotic phenylalanyl-tRNA synthetases. The discrimination, established by this mechanism, is clearly not sufficient to achieve the necessary fidelity. Thus, the specificity must necessarily be enhanced by use of a second discriminatory step subsequent to initial binding/activation. There are, however, differences in the strategy to achieve accuracy for phenylalanyl-tRNA synthetases from a eubacterial, archaebacterial, lower (yeast, *N. crassa*), and higher (turkey) eukaryotic organism including their mitochondria (Gabius et al., 1983a,b; Rauhut et al., 1985).

With bean cytoplasmic enzyme, natural noncognate amino acids are not detectable as stable aminoacyl-$tRNA^{Phe}$-C-C-A(3′NH_2). Neither transfer of amino acid to $tRNA^{Phe}$ lacking the nonaccepting 3′-OH group nor transient transfer to $tRNA^{Phe}$-C-C-A but AMP production was measurable. This pretransfer proofreading (Jakubowski & Fersht, 1981) resembles the mechanism of enzymes from the two liver compartments and from an archaebacterium. With the chloroplastic enzyme, natural amino acids (tyrosine, methionine) are transferred to $tRNA^{Phe}$-C-C-A(3′NH_2). This posttransfer proofreading resembles the mechanism of enzymes from eubacteria and from the two yeast compartments. A detailed comparative list of all phenylalanyl-tRNA synthetases studied in this respect is given in Table IV.

The AMP production is based on intermediate activation and indicative for corrective steps. The cytoplasmic enzyme seems to use different modes of discrimination against natural amino acids within the pretransfer proofreading mechanism. While methionine-dependent AMP production was not affected by tRNA, the tyrosine-dependent process was slightly inhibited in the presence of $tRNA^{Phe}$-C-C-3′dA. AMP production in the presence of modified $tRNA^{Phe}$s thus reveals no significant implication of the terminal adenosine to hydrolysis of noncognate natural amino acids. This is in contrast to the liver enzymes, where pretransfer proofreading for natural amino acids is dependent on the 3′-OH group of the terminal adenosine of the $tRNA^{Phe}$.

Table IV: Discrimination against Noncognate Natural Amino Acids by Different Phenylalanyl-tRNA Synthetases[a]

source of enzyme	initial discrimination[b]	proofreading mechanism[c]	proofreading capacity
archaebacterial	++++	pre	+++
eubacterial	+++	post	+
lower eukaryote (cyto)	++	post	++
lower eukaryote (mito)	+++	post	++
vertebrate (cyto)	+	pre	+++
vertebrate (mito)	++	pre	++++
plant (cyto)	++	pre	++++
plant (chloro)	+	post	++

[a] Gabius et al. (1983a,b), Rauhut et al. (1985), and this paper. [b] Reflected in the V_{Phe}/V_{Xxx} values of ATP/PP_i exchange reaction in the absence of tRNA. [c] In terms of pretransfer and posttransfer proofreading. (+) to (++++) indicates the increasing degrees.

Two plant-specific instances may probably shed some light on the physiological significance of the described mechanisms of accuracy that demonstrates that simple extrapolations for different organisms and even within higher eukaryotes are not generally valid. In contrast to other studied sources of phenylalanyl-tRNA synthetases, higher plants synthesize a great variety of amino acids structurally similar to those amino acids found in proteins (Bell, 1972; Fowden et al., 1979). To prevent possible toxic effects, e.g., by incorrect incorporation into protein, the discrimination against these analogues places an evolutionary constraint especially on plant cytoplasmic aminoacyl-tRNA synthetases, as is further exemplified by Lea & Norris (1977). It is also interesting to note that in plants the availability of intact tRNA changes with different growth stages. Whereas the specific activity for cytoplasmic phenylalanyl-tRNA synthetase remains constant during germination in the case of bean and pea (Anderson & Fowden, 1969), in lupin seed and wheat embryos the level of cytoplasmic tRNA with partly degraded CCA terminus drastically decreases during germination in relation to increase of ATP content that may have a common regulatory function in germinating seeds (Dziegielewski et al., 1979). Under these conditions, the efficient enzymatic hydrolysis of noncognate aminoacyl adenylates may strongly contribute to the rejection of noncognate amino acids, as was described earlier for the lupin valyl-tRNA synthetase (Jakubowski, 1980).

Analysis of the divergence of enzymatic properties among different species and their intracellular compartments can be helpful to gain insights on the history of life and on forces that drive evolution. Considering the architecture of the active site of phenylalanyl-tRNA synthetases, cross-reactivity with tRNA suggests differences in this part. Whereas the cytoplasmic enzyme does not aminoacylate eubacterial or chloroplastic tRNA, the chloroplastic enzyme uses these types of tRNAs, but not cytoplasmic tRNA, as a substrate (Jeannin et al., 1978; Rauhut et al., 1984). The specificity to ATP in contrast to the amino acid is maintained at the binding level and does not require further corrective steps (Gabius et al., 1982; Rauhut et al., 1984). Species-specific mechanistic differences are mainly detected by using phenylalanine analogues. Thus, the subtle interplay between the active site of the enzyme, the amino acid, and conformational changes induced by complexation with tRNA can result in different ways for dis-

crimination of phenylalanine analogues (Cramer & Gabius, 1984).

In this respect the fundamental difference in the proofreading mechanisms of the two plant enzymes deserves special attention. A similar analysis for the enzymes from mitochondria of fungi and animals led to the conclusion that these two heterotopic intracellular isoenzymes are formed by gene duplication of the nuclear gene (Gabius et al., 1983a). This conclusion appears rather unlikely in the case of the origin of the chloroplastic phenylalanyl-tRNA synthetase. In contrast to a report on chloroplastic rye triosephosphate isomerase that appears to have evolved from a duplication of the nuclear gene (Kurzok & Feierabend, 1984), our data appear tentatively consistent with a model that proposes the transfer of a gene for the plastid-specific enzyme, originally derived from the genome of an eubacterial endosymbiont, to the nucleus during evolution of the plant cell (Weeden, 1981). Support for this proposed model of displaced genes has come from an enzyme in carbohydrate metabolism, phosphoglucose isomerase (Weeden et al., 1982). Other constituents of protein biosynthesis, the tRNAs and rRNAs, that are coded by the plastid genome appear to cluster within the eubacterial lineage in sequence comparisons (Eigen & Winkler-Oswatitsch, 1981; McCarroll et al., 1983; Gray et al., 1984). The chloroplastic phenylalanyl-tRNA synthetase thus gives an evolutionary important example of a nuclear coded participant of protein biosynthesis to exhibit eubacterial features.

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Registry No. Phe, 63-91-2; Tyr, 60-18-4; Leu, 61-90-5; Met, 63-68-3; *p*-fluorophenylalanine, 51-65-0; β-phenylserine, 1078-17-7; 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.

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