Evolutionary Aspects of Accuracy of Phenylalanyl-tRNA Synthetase. Accuracy of Fungal and Animal Mitochondrial Enzymes and Their Relationship to Their Cytoplasmic Counterparts and a Prokaryotic Enzyme†

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ABSTRACT: Phenylalanyl-tRNA synthetases from mitochondria of yeast and hen liver resemble their corresponding cytoplasmic counterparts. Whereas slight intraspecies differences at the amino acid binding site, reflecting variations in the structures of these distinct enzymes, are exploitable by phenylalanine analogues, no intraspecies difference can be noted for the strategies to achieve the high fidelity of protein synthesis. While the yeast mitochondrial enzyme follows the pathway of posttransfer proofreading, the hen liver mitochondrial enzyme uses a tRNA-dependent pretransfer proofreading in the case of the natural amino acids. The accuracy of mitochondrial phenylalanyl-tRNA synthetases appears to be even better than the accuracy of the corresponding cytoplasmic enzymes. Interspecies rather than intraspecies differences for the functional role of certain amino acid residues of the enzymes further indicate the close relationship of the intracellular heterotopic isoenzymes. By use of a highly sensitive immunospotting procedure, common antigenic determinants are detected only within the enzymes from the two intracellular compartments of the same organism. The results suggest the origin of the cytoplasm–mitochondrion isoenzyme pair by independent gene duplication of the ancestral nuclear gene. A similarity of mitochondrial enzymes to the phenylalanyl-tRNA synthetase from *Escherichia coli* is not observed.

The ability of mitochondria for protein biosynthesis is well established. They therefore contain a set of mitochondrial aminoacyl-tRNA synthetases that are also coded for by the nuclear genome but often differ in chromatographic mobility during the isolation procedure from their cytoplasmic counterparts, suggesting nonidentity (Chiu & Suyama, 1973; Schneller et al., 1976; Schneller et al., 1978; Diatewa & Stahl, 1980; Felter et al., 1981; Gabius & Cramer, 1982a).

Since we have shown for phenylalanyl-tRNA synthetases from a prokaryotic organism and the cytoplasm from lower and higher eukaryotic organisms that they reveal obvious mechanistic differences in their strategy to achieve the necessary fidelity (Gabius et al., 1983a), it is of special interest for the description of this aspect of mitochondrial protein biosynthesis and evolutionary considerations to extend these studies to mitochondrial phenylalanyl-tRNA synthetases. As fungal and animal mitochondria exhibit strikingly different patterns of gene organization and transcription (Gray & Doolittle, 1982), both pairs of intracellular heterotopic isoenzymes, from yeast and hen liver, are therefore examined. Besides the description of the interaction of phenylalanine analogues with the mitochondrial enzymes and the mechanistic implications, the role of certain amino acid residues in the catalytic activity of the phenylalanyl-tRNA synthetases from *Escherichia coli* and the two different intracellular compartments of yeast and hen liver is comparatively studied by chemical modification with different types of reagents. As an approach to furthermore detect structural homologies between the different enzymes and to examine the relatedness of the mitochondrial to cytoplasmic and prokaryotic enzymes, different immunological procedures with antibodies against the different phenylalanyl-tRNA synthetases were employed. A high degree of conservation of structural domains has been noted for other enzymes with nucleic acids as substrates, e.g., RNA and DNA polymerases (Huet et al., 1982; Chang et al., 1982). This comparative functional and structural analysis leads to conclusions on the origin of the cytoplasm–mitochondrion isoenzyme pair and completes our studies on the elucidation of the mechanism of phenylalanyl-tRNA synthetases to achieve the necessary fidelity in organisms from different branches of the evolutionary tree and their intracellular compartments.

Experimental Procedures

Materials. $tRNA^{Phe}$-C-C and $tRNA^{Phe}$-C-C-A were isolated by the procedure of Schneider et al. (1972) from unfractionated bakers' yeast tRNA (Boehringer Mannheim, FRG). Incorporation of 3′-deoxy-3′-aminoadenosine 5′-phosphate into $tRNA^{Phe}$-C-C was performed by using tRNA nucleotidyltransferase (EC 2.7.7.25), kindly provided by Dr. H. Sternbach, as described previously (Sprinzl & Sternbach, 1979). tRNA from yeast and hen liver mitochondria was isolated essentially as described previously (Martin et al., 1978). Phenylalanyl-tRNA synthetases (EC 6.1.1.20) were purified to homogeneity: from *E. coli* according to Holler et al. (1975), from yeast cytoplasm by the $tRNA^{Phe}$-induced solubility shift in salting-out chromatography as described by von der Haar (1978), from yeast mitochondria as described by Diatewa & Stahl (1980), using affinity elution with $tRNA^{Phe}$ as a further step, from hen liver cytoplasm as described by Gabius et al. (1983b), and from hen liver mitochondria as described by Gabius & Cramer (1982a). Enzyme preparations from different vertebrate livers were prepared as described previously (Gabius et al., 1982a). Proteinase K (EC 3.4.21.14) was from Boehringer Mannheim (FRG). ^{14}C-Labeled amino acids of specific activity 521 mCi/mmol, [^{14}C]ATP of specific activity 55 mCi/mmol, [^{32}P]pyrophosphate of specific activity 11.3 mCi/mmol, and Bolton–Hunter reagent were purchased from Radiochemical Centre (Amersham, England). Phenylalanine (L and D), diethyl pyrocarbonate, and 3-(2-ethyl-5-isooxazolio)benzenesulfonate were obtained from Merck (Darmstadt, FRG); recrystallized tyrosine, methionine, leucine, *p*-fluorophenylalanine, β-phenylserine, ochratoxin A [7-carboxyl-5-chloro-8-hydroxy-3,4-dihydro-3(*R*)-methylisocoumarin linked by its 7-carboxyl

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group to L-phenylalanine], and horse radish peroxidase, type VI, were from Sigma (Munich, FRG); β-thien-2-ylalanine, mimosine [β-(1,4-dihydro-3-hydroxy-4-oxo-1-pyridyl)alanine], and rose bengal were purchased from Serva (Heidelberg, FRG); phenyl phosphorodichloridate was from Ega (Steinheim, FRG); pyridoxal 5′-phosphate and *p*-(chloromercuri)-benzenesulfonic acid were from Calbiochem (Giessen, FRG); protein A, protein A–Sepharose CL-4B, and *N*-succinimidyl 3-(2-pyridyldithio)propionate were from Pharmacia (Freiburg, FRG); 2-amino-4-methylhex-4-enoic acid was a gift from Dr. L. Fowden (London); *N*-benzyl-L- or -D-phenylalanine was synthesized from nonradioactive phenylalanine 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 previously (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 hydrochloride (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 = 60 000 cpm). The incorporation of ^{14}C-labeled amino acid was measured as described previously (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 previously (Igloi et al., 1977; Gabius et al., 1983a). 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 periodic 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, 3MM), and immediately quenched with trichloroacetic acid.

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 specific activity 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 30 000 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).

Checking 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 (Fersht, 1977) is given by

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

From amino acid pool data given by Ward & Mortimore (1978) and Davidova et al. (1981), 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 (1977):

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

Chemical Modification of Phenylalanyl-tRNA Synthetases. Chemical modification was performed at 0 °C by treatment of the enzymes with the modifying reagent for certain periods of time. To measure the activity of the modified enzymes, aliquots were immediately diluted in the test mix. Treatment with diethyl pyrocarbonate was performed in 50 mM Tris-HCl buffer (pH 7.0) or in 50 mM potassium phosphate buffer (pH 6.0) containing 0.2 mM dithioerythritol, 0.2 mM ethylenediaminetetraacetic acid (EDTA), and 0.2 mM $MgCl_2$. Diethyl pyrocarbonate was added in absolute ethanol. The final ethanol concentration was less than 2%, which was without any effect on enzyme activity. The differential spectra of carboethoxylated vs. untreated enzyme with or without hydroxylamine treatment were obtained by measurements in a Zeiss PMQ II spectrophotometer. Reactivation of enzyme activity of the modified enzymes by hydroxylamine (0.25 M) was studied after 6 h of incubation at 4 °C in 50 mM Tris-HCl buffer (pH 7.0), 5 mM $MgCl_2$, 50 mM KCl, 0.2 mM dithioerythritol, 0.2 mM EDTA, and 50 μg/mL bovine serum albumin. Reversible modification of thiol groups before diethyl pyrocarbonate treatment was performed in thiol-free 50 mM potassium phosphate buffer (pH 6.0) containing 0.2 mM EDTA and 0.2 mM $MgCl_2$ with 75 μM *p*-(chloromercuri)-benzenesulfonic acid for 1 h at 15 °C. This treatment results in nearly total loss of activity for all enzymes. The activity is restored by treatment with 5 mM dithioerythritol for 10 min at 37 °C. Photooxidation in the presence of 0.2 mM rose bengal was carried out at 20 °C with a 2500-W lamp at 15-cm distance in 50 mM potassium phosphate buffer (pH 7.0), 10 mM $MgCl_2$, 0.1 mM 2-mercaptoethanol, and 0.1 mM EDTA

with 1.0 μM enzyme. Treatment with phenyl phosphorodichloridate and 3-(2-ethyl-5-isooxazolio)benzenesulfonate was conducted in 50 mM Tris-HCl buffer (pH 7.0). Modification by pyridoxal 5′-phosphate, followed by reduction with borohydride, was done in 50 mM potassium phosphate buffer (pH 7.0) at 25 °C. For the preparation of inactivated enzyme in quantitative scale to determine the number of modified residues, the reaction was stopped by adding an amino acid (histidine, serine, glutamic acid, lysine) to a final concentration of 20 mM after certain periods of time. The excess reagent and amino acid were removed by gel filtration on a Sephadex G-50 column. The concentrations of reagents were calculated to give a similar molar excess of reagent with respect to the content of amino acid residues (histidine, lysine) of the enzymes, as given by amino acid analysis (Kosakowski & Böck, 1970; Gabius & Cramer, 1982b; H.-J. Gabius et al., unpublished results). Since none of the reagents for modifications were commercially available as a radioactive substance, amino acid analyses after enzymatic or acidic hydrolysis were performed as previously described (Gabius & Cramer, 1982b) for determination of the number of modified amino acid residues.

Immunological Procedures. Aliquots containing 150 μg of each native phenylalanyl-tRNA synthetase were emulsified with complete Freund's adjuvant and intradermally injected into rabbits. After 3 weeks, booster injections of 50 μg were given in incomplete Freund's adjuvant, and postboost bleeding was taken 1 week thereafter. The immunoglobulin G (IgG) fraction thus obtained was purified by chromatography on protein A–Sepharose CL-4B. The number of antigenic determinants for all enzymes with F_{ab} fragments was determined according to Scheinker et al. (1979). Ouchterlony double diffusion and immunoelectrophoresis was carried out on microscope slides coated with 1% agarose in 40 mM Tris-HCl (pH 8.5) buffer containing 20 mM sodium acetate and 1 mM EDTA. Diffusion was carried out in a moist chamber for 48 h at room temperature. The slides were washed for 24 h with several changes of buffer containing 1% NaCl and for 4 h with four changes of distilled water and then air-dried. Immunoelectrophoresis was run under the same conditions at 10 mA for 40 min. After electrophoresis, IgG solutions were added to the antisera trough, and diffusion was allowed to take place in a moist chamber overnight at room temperature. The precipitin lines were visualized by staining with amidoblack. For immunoinactivation, aliquots of purified enzymes were incubated at 37 °C for 15 min with increasing amounts of immune serum in the aminoacylation medium. The residual activity was determined by measuring the aminoacylation of tRNA added to the reaction mixture. Control experiments performed with only preimmune serum showed no immunoinactivation of the different phenylalanyl-tRNA synthetases. Electrophoretic blotting and detection of cross-reactivity with fluorescein-conjugated goat anti-rabbit IgG (Western blot immunodetection) were done as described (Towbin et al., 1979).

For the immunospotting procedure, 1 μL of phenylalanyl-tRNA synthetase or extract was spotted on dry pieces of nitrocellulose (Sartorius SM11306, Göttingen, FRG) of about 1 cm^2, and the filters were placed in tissue-culture plates. The protocol for incubation in phosphate-buffered saline (5.8 mM phosphate, pH 8.0, 140 mM NaCl, 3 mM KCl) containing 3% bovine serum albumin (1 h at 37 °C) for saturation of protein binding sites, washing with the same buffer containing 0.5% bovine serum albumin, and incubation with this buffer containing 0.2% Triton X-100, 0.2% sodium dodecyl sulfate, and 20 μg/mL of purified immunoglobulins (1 h at 37 °C) followed the procedure of Huet et al. (1982) with slight modifications. To remove unbound immunoglobulins, the filters are washed with 1 mL of the above buffer with three rapid buffer changes and three changes after 10 min. After being washed, the filters were then incubated for 1 h at 37 °C in 0.5 mL of the same buffer containing either protein A–peroxidase monoconjugate (50 μg/mL) prepared according to Surolia & Pain (1981) or ^{125}I-labeled protein A (1.2×10^6 cpm/mL), prepared with a specific activity of 150 Ci/mmol according to Langone et al. (1977). The filters are then rapidly washed with three changes and then 3 times for 10 min at room temperature with 1 mL of the same buffer without protein A. Treatment with ^{125}I-labeled protein A was followed by determination of radioactivity in a γ-counter. The monoconjugate-treated filters were incubated in a freshly prepared solution of 3,3′-diaminobenzidine or 4-chloronaphthol (1 mg/mL, 0.01% H_2O_2) in 50 mM phosphate buffer (pH 6.0), and the reaction was terminated after sufficient brownish color developed (5–10 min and up to 1 h, respectively) by in water washing and fixation in a mixture of 5% 2-propanol and 7.5% acetic acid in 50 mM phosphate buffer, pH 6.0. After they were dry, the filters are scanned on a Helena Quick Scan R&D densitometer set up in the reflection mode. Treatment with paraffin oil enables scanning in the absorption mode with a normal densitometric scanning device. Even a simple visual comparison of the intensity of the spot in question with a dilution series of the standard leads to almost the same result. Preparation of ^{125}I-labeled phenylalanyl-tRNA synthetases (sp act. 1200–2000 Ci/mmol) and the radioimmunoassay were performed as described (Behra et al., 1981).

Results

Substrate Properties of Phenylalanine Analogues. The mitochondrial phenylalanyl-tRNA synthetases do not activate all tested analogues, as was recently shown for their cytoplasmic counterparts and the *E. coli* enzyme (Gabius et al., 1983a). *N*-Benzyl-D-phenylalanine is not a substrate in the ATP/PP_i pyrophosphate exchange for both mitochondrial enzymes, whereas ochratoxin A is activated only by the mitochondrial enzyme from yeast (Table I). Problems of contamination in either the enzyme preparation or in the amino acid that can lead to misinterpretation of the data were shown to be negligible following the scheme of Igloi et al. (1979). In general, the reactions with noncognate amino acids are characterized by lower rates (compared to phenylalanine) and higher K_m values. These parameters allow one to calculate the relative specificity against the noncognate amino acids, expressed as V_{Phe}/V_{Xxx} (see Experimental Procedures). As a first indication for the relative specificity of mitochondrial phenylalanyl-tRNA synthetases, these data demonstrate a relatively better discrimination against noncognate amino acids compared to the cytoplasmic enzymes that even surpasses the specificity of the *E. coli* enzyme in the case of β-phenylserine and *N*-benzyl-L-phenylalanine (Gabius et al., 1983a). In the case of discrimination against a natural amino acid, leucine for example (with respect to in vivo amino acid concentrations), the specificities are 2400:1 and 1800:1 for the mitochondrial enzymes from yeast and hen liver compared to 360:1, 4:1, and 7400:1 for the cytoplasmic enzymes from yeast and hen liver and the *E. coli* enzyme. This level of discrimination alone, though relatively more accurate than for the corresponding cytoplasmic enzyme, 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.

Table I: Substrate Properties of Tested Amino Acids for Phenylalanyl-tRNA Synthetase from Yeast Mitochondria (Scm) and Hen Liver Mitochondria (hlm)[a]

amino acid	K_m (μM)		k_{cat} (min^{-1})		V_{Phe}/V_{Xxx}[b]		V_{Phe}/V_{Xxx}[c]		$\Delta\Delta G_B$ (kJ/mol)	
	Scm	hlm	Scm	hlm	Scm	hlm	Scm	hlm	Scm	hlm
phenylalanine (Phe)	34	38	320	75						
	11	12	112	27						
tyrosine (Tyr)	1250	30000	29	70	410	86	600	64	15.5	11.5
	1400	*d*	2.6	*d*	5480	*d*	7880	*d*	22.1	*d*
leucine (Leu)	2000	8000	11	9	1710	1790	2400	1800	19.2	19.3
	d	*d*	*d*	*d*	*d*	*d*	*d*	*d*	*d*	*d*
methionine (Met)	840	4000	16	11	470	710	500	*d*	15.8	16.9
	1700	*d*	1	*d*	17300	*d*	18400	*d*	25.2	*d*
p-fluorophenylalanine	77	64	148	40	5	3			4.1	2.9
	175	280	80	24	22	26			8.0	8.4
β-phenylserine	238	340	63	12	36	56			9.2	10.4
	340	840	42	23	83	83			11.4	11.4
β-thien-2-ylalanine	175	210	95	34	17	12			7.4	6.5
	142	600	68	19	22	70			8.0	10.9
2-amino-4-methylhex-4-enoic acid	910	98	78	19	104	10			12.0	5.9
	338	740	20	4	170	420			13.2	15.5
mimosine	10000	9000	22	6	4270	2940			21.5	20.6
	d	*d*	*d*	*d*	*d*	*d*			*d*	*d*
N-benzyl-L-phenylalanine	2500	2500	13	12	1880	410			19.4	15.5
	2900	8000	4	1	7300	1900			22.9	25.4
N-benzyl-D-phenylalanine	*d*	*d*	*d*	*d*	*d*	*d*			*d*	*d*
	d	*d*	*d*	*d*	*d*	*d*			*d*	*d*
ochratoxin A	1100	*d*	2	*d*	5220	*d*			22.1	*d*
	d	*d*	*d*	*d*	*d*	*d*			*d*	*d*

[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] With consideration of in vivo concentration. [d] No substrate.

In the presence of tRNA, the changes of the kinetic parameters for the aminoacylation of tRNA^{Phe}-C-C-A(3′NH$_2$), where the potentially labile ester bond of the aminoacyl-tRNA^{Phe}-C-C-A is replaced by an amide bond with phenylalanine analogues, indicate that the binding of the amino acid is more specific with the binary enzyme–tRNA complex than with the free enzyme, as expressed in terms of V_{Phe}/V_{Xxx} (Table I). These values are experimentally confirmed by measuring the excess of analogue necessary to produce a 50% inhibition of phenylalanine aminoacylation. No transfer is seen for the larger analogue mimosine and for *N*-benzyl-D-phenylalanine and ochratoxin A.

By use of a back-titration (see Experimental Procedures), the amount of residual non-aminoacylated tRNA^{Phe}-C-C-A or tRNA^{Phe}-C-C-A(3′NH$_2$) after preincubation of the enzyme with nonradioactive amino acid is determined by the extent of [^{14}C]phenylalanine incorporation. The noncognate natural amino acids are not detectable as aminoacyl-tRNA^{Phe}-C-C-A for both enzymes and as aminoacyl-tRNA^{Phe}-C-C-A(3′NH$_2$) for the hen liver mitochondrial enzyme, as was noted for the corresponding cytoplasmic enzymes. One difference appears for the yeast enzymes. Leucine is not transferred to tRNA^{Phe}-C-C-A(3′NH$_2$) by the mitochondrial enzyme (Table II, columns 2–5). Another intraspecies difference is also noted for the hen liver enzymes, because only the mitochondrial enzyme transfers 2-amino-4-methylhex-4-enoic acid to tRNA^{Phe}-C-C-A(3′NH$_2$) and tRNA^{Phe}-C-C-A.

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, enzyme–aminoacyl adenylate complex with [^{14}C]tyrosine as amino acid was prepared in situ. The transfer of amino acid, transiently coupled to tRNA^{Phe}-C-C-A upon mixing tRNA^{Phe} and the enzyme–[^{14}C]tyrosyl adenylate complex, only takes place for the mitochondrial yeast enzyme, not for the mitochondrial liver enzyme (Figure 1). The results are identical for yeast cytoplasmic tRNA^{Phe} and the corresponding mitochondrial tRNA^{Phe}s, suggesting a lack of influence of the

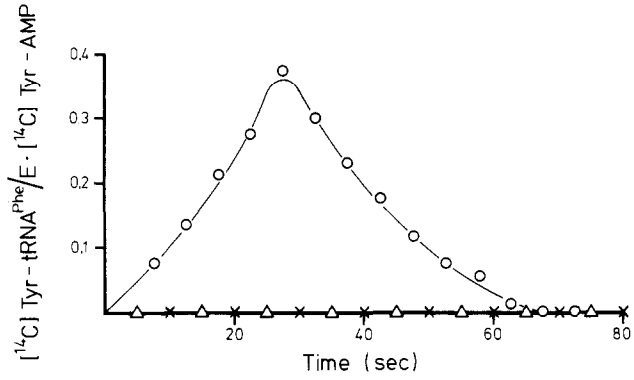

FIGURE 1: Transient formation of ^{14}C-labeled aminoacyl-tRNA^{Phe}-C-C-A during rejection of phenylalanine analogues by the phenylalanyl-tRNA synthetases of mitochondria from yeast and hen liver at pH 7.65 and 0 °C. Enzyme–^{14}C-labeled aminoacyl-AMP (2.8 μM) was formed in situ in the presence of inorganic pyrophosphatase. Transfer of [^{14}C]tyrosine to tRNA^{Phe} (7 μM) by yeast (○) and hen liver (×) mitochondrial phenylalanyl-tRNA synthetase. Transfer of [^{14}C]tyrosine to tRNA^{Phe} (7 μM) by hen liver mitochondrial enzyme with tRNA^{Phe}-C-C-A(3′NH$_2$) (△). Aliquots were spotted onto paper filter disks and immediately quenched with trichloroacetic acid.

source of tRNA in this reaction. Thus the transfer of amino acid to the tRNA^{Phe}-C-C-A(3′NH$_2$) used in quantitating aminoacylation is not an artifact. As a further control, attempts to trap any [^{14}C]Tyr-AMP dissociated from the enzyme, as outlined by Gabius et al. (1983a), were unsuccessful. These results closely resemble the data for the cytoplasmic enzymes from both organisms.

In the course of aminoacylation reaction, ATP is hydrolyzed to AMP. A nonstoichiometric amount of AMP production relative to the charging reaction is therefore an indication that a 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, columns 6 and 7) can be

Table II: Further Substrate Properties of the Tested Amino Acids

	aminoacylation of $tRNA^{Phe}$-C-C-A(3′NH_2) (%)[a]		aminoacylation of $tRNA^{Phe}$ (%)[b]		[^{14}C]AMP production, k_{cat} (min^{-1})[c]		k_{cat}[$tRNA^{Phe}$-C-C-A(3′NH_2)]/k_{cat}(AMP production)[d]	
	Scm	hlm	Scm	hlm	Scm	hlm	Scm	hlm
phenylalanine (Phe)	100	100	100	100	6.8	4.0	15.4	6.8
tyrosine (Tyr)	85	*e*	*e*	*e*	1.67	3.4	1.02	*e*
leucine (Leu)	*e*	*e*	*e*	*e*	*e*	*e*	*e*	*e*
methionine (Met)	70	*e*	*e*	*e*	0.62	1.7	0.74	*e*
p-fluorophenylalanine	99	96	82	86	5.3	2.6	13.1	9.7
β-phenylserine	99	96	62	40	1.13	4.7	32.2	2.6
β-thien-2-ylalanine	94	98	10	10	56.7	7.5	1.2	1.5
2-amino-4-methylhex-4-enoic acid	96	94	7	4	11.5	5.6	1.7	1.1
mimosine	*e*	*e*	*e*	*e*	*e*	1.5	*e*	*e*
N-benzyl-L-phenylalanine	3	10	*e*	<1	0.27	1.4	1.3	0.7
N-benzyl-D-phenylalanine	*e*	*e*	*e*	*e*	*e*	0.2	*e*	*e*
ochratoxin A	*e*	*e*	*e*	*e*	*e*	*e*	*e*	*e*

[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 non-stoichiometric 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. [e] No substrate.

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

enzyme	source of mitochondria	k_{cat} (min^{-1}) Phe	Tyr	Met
phenylalanyl-tRNA synthetase	bakers' yeast	<0.05	<0.05	<0.05
	hen liver	0.61	0.4	0.2
E·$tRNA^{Phe}$-C-C	bakers' yeast	<0.1	<0.05	<0.05
	hen liver	0.63	0.4	0.2
E·$tRNA^{Phe}$-C-C-3′dA	bakers' yeast	<0.1	<0.05	<0.05
	hen liver	1.54	1.03	0.51
E·$tRNA^{Phe}$-C-C-A	bakers' yeast	6.8	1.67	0.62
	hen liver	4.0	3.4	1.7
E·$tRNA^{Phe}$-C-C-2′dA	bakers' yeast	<0.1	<0.05	<0.05
	hen liver	3.6	3.3	1.67
E·$tRNA^{Phe}$-C-C-A(3′NH_2)	bakers' yeast	0.52	0.12	<0.1
	hen liver	1.71	0.97	0.42

compared to the turnover number for aminoacylation of $tRNA^{Phe}$-C-C-A(3′NH_2), when a transfer has occurred. This quotient (Table II, columns 8 and 9) is a measure of the proofreading capacity of a synthetase by indicating the number of aminoacylations per proofreading step. The low ratio of synthetic to the subsequent hydrolytic step for the mitochondrial enzymes clearly demonstrates that their first selection is followed by an enhancement of accuracy that again surpasses the accuracy of the corresponding cytoplasmic enzyme. This quotient is also significantly smaller than that for the *E. coli* enzyme, which is characterized by a high specificity in the initial recognition. It is interesting to note the similarities between the pairs of enzymes from one organism. The gradation of the quotients for the analogues is similar and includes the fact that the hydrolysis of β-phenylseryl-$tRNA^{Phe}$ is less than that for phenylalanine (32 to 15 and 46 to 30 as expressed in the ratio of synthetic to subsequent hydrolytic step) for the mitochondrial and cytoplasmic yeast enzymes but higher for the hen liver enzymes (6.8 to 2.6 and 8.2 to 6.6). A different binding of this analogue and positioning at the active site is therefore due to both hen liver enzymes. The values for the quotient presented in Table II (columns 8 and 9) are consistent with the stoichiometry of Phe-$tRNA^{Phe}$-C-C-A formation of the yeast and hen liver mitochondrial enzymes: 0.93 and 0.86 nmol of Phe-$tRNA^{Phe}$/nmol of ATP.

The role played by the tRNA in AMP production that is nonstoichiometric with respect to aminoacylation and indicative for the extent of proofreading is determined by using $tRNA^{Phe}$s modified at the accepting 3′-terminal adenosine (Table III). The results for the mitochondrial yeast enzyme reveal close mechanistic similarity to the corresponding cytoplasmic enzyme, including the importance of the 3′-hydroxyl group for proofreading. With the enzyme–$tRNA^{Phe}$-C-C-2′dA complex that is not a substrate for phenylalanylation, an efficient proofreading takes place, and the nonstoichiometric ATP hydrolysis with respect to aminoacylation of $tRNA^{Phe}$-C-C-A(3′NH_2) suggests that a small amount of deacylation must also occur for this enzyme from the 2′-acceptor OH prior to transacylation to the 3′-NH_2. Like the cytoplasmic enzyme from hen liver, the mitochondrial enzyme transfers phenylalanine, no other natural amino acids, but some analogues like β-thien-2-ylalanine and *N*-benzyl-L-phenylalanine to $tRNA^{Phe}$-C-C-3′dA. A difference to the cytoplasmic enzyme is only noted for the higher rate of AMP production in the presence of the nonaccepting $tRNA^{Phe}$-C-C-2′dA and phenylalanine. This substantiates that kinetic parameters and mechanisms of proofreading indicate a close relationship for the enzymes of these intracellular compartments of the same organism.

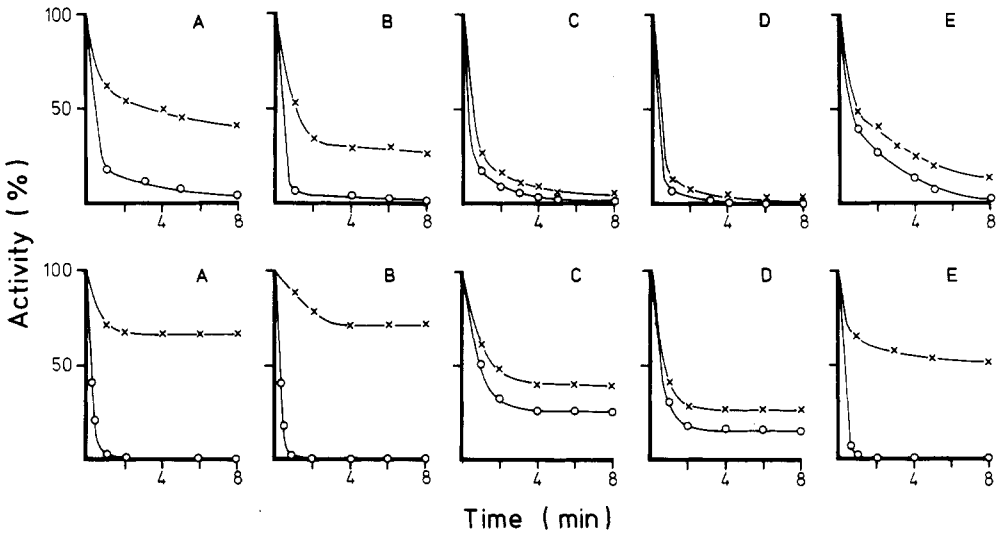

FIGURE 2: (Top row) Influence of diethyl pyrocarbonate treatment on activities of ATP/PP$_i$ pyrophosphate exchange (×) and aminoacylation (○) of phenylalanyl-tRNA synthetases from hen liver cytoplasm (A), hen liver mitochondria (B), yeast cytoplasm (C), yeast mitochondria (D), and *E. coli* (E). A 50-fold excess of diethyl pyrocarbonate, given as molar ratio to total histidine content of the enzyme, was added at 0 °C to absolute protein concentrations of 120–200 μg/mL and incubated for indicated periods of time before assaying. (Bottom row) Influence of phenyl phosphorodichloridate treatment on the activities of ATP/PP$_i$ pyrophosphate exchange (×) and aminoacylation (○) of phenylalanyl-tRNA synthetases as given above. Absolute protein concentrations of 120 μg/mL for all enzymes were treated with 1.6 mM reagent at 0 °C and incubated for indicated periods of time before assaying.

Comparison of Effects of Chemical Modifications with Reagents of Different Specificity. Comparison of the effects of modification of certain amino acid residues to detect relatedness among homologous enzymes (Bodo & Foucault, 1982) allows insights into their possible functional role in the enzymatic reaction, therefore extending the functional studies on kinetic parameters and proofreading mechanisms.

Treatment of the enzymes with diethyl pyrocarbonate, which ethoxyformylates histidine residues under the described conditions, results in inhibition of ATP/PP$_i$ pyrophosphate exchange and tRNA aminoacylation (Figure 2, top row). Similar observations have already been reported only for the *E. coli* and the cytoplasmic yeast enzyme (Hennecke & Böck, 1974; Raffin & Remy, 1978). In the case of the *E. coli* and both yeast enzymes, the inhibition of both enzymatic activities is parallel. tRNA aminoacylation by the hen liver enzymes, however, is much more sensitive to modification than ATP/PP$_i$ pyrophosphate exchange, the reaction characterizing the amino acid activation step. Reduction in AMP production and hydrolysis of preformed [^{14}C]Phe-tRNA^{Phe} closely parallel the decrease in tRNA^{Phe} aminoacylation. The specificity of histidine modification under the described conditions is confirmed as follows: nearly complete reactivation of the enzymes after 6 h of incubation at 4 °C with 0.25 M hydroxylamine; concomitant disappearance of the differential absorbance around 242 nm that is characteristic for N^{im}-(carboethoxy)histidines (Ovadi et al., 1967; Morris & McKinley-McKee, 1972); no change in the absorbance at 278 nm after modification excluding tyrosine modification (Mühlrad et al., 1967; Burstein et al., 1974); no influence of reversible masking of thiol groups by *p*-(chloromercuri)benzenesulfonic acid before treatment with diethyl pyrocarbonate on the inactivation kinetics of all enzymes; a similar pattern of inactivation with rose bengal, another reagent with apparent histidine specificity (Westhead, 1965) (not shown). As judged by the increase of the absorption at 242 nm, modification of four to six histidine residues per tetrameric enzyme is sufficient for 50% loss of aminoacylation activity in the absence of substrates. One of the possible reasons for inactivation of phenylalanyl-tRNA synthetases may be impaired ability to bind tRNA^{Phe}. The ability of the modified enzyme preparations to form a complex with tRNA^{Phe} was tested by nitrocellulose filter assays after addition of [^{14}C]Phe-tRNA^{Phe}. No significant change of the results to controls with unmodified enzymes can be detected. This is in contrast to the beef pancreas tryptophanyl-tRNA synthetase, where the modified enzyme looses its ability to bind tRNA^{Trp} by carbethoxylation of histidine residues (Favorova et al., 1978). The imidazole ring of histidine residues may therefore strongly participate in substrate binding or directly in the catalytic process of activation, predominantly of the yeast enzymes.

Phenyl phosphorodichloridate is known to react with strong nucleophilic groups like hydroxyl and amino groups (Wins & Wilson, 1974). The enzymatic activities also differ in their sensitivity to inhibition (Figure 2, bottom row). A preferential inactivation of the aminoacylation reaction is noticed for the phenylalanyl-tRNA synthetases from *E. coli* and the hen liver enzymes. A more parallel pattern of inhibition of ATP/PP$_i$ pyrophosphate exchange and aminoacylation reaction is revealed for the yeast enzymes, where serine residues appear to be important primarily for substrate binding and activation. Modification of 3.7 ± 0.6 serine residues (mean ± SD for six determinations), as indicated by amino acid analyses after enzymatic hydrolysis, leads to a 50% decrease in aminoacylation and AMP-producing activities for the hen liver enzymes and the *E. coli* enzyme, while at least five serine residues appear to be modified in the yeast enzymes. Besides serine residues, threonine residues are also accessible to modification by phenyl phosphorodichloridate with prolonged incubation time, thus exerting no influence on the rapid activity decrease.

For the selective modification of protein carboxyl groups, reaction with 3-(2-ethyl-5-isoxazolio)benzenesulfonate was employed. It exerts no influence on the enzymatic activities of the phenylalanyl-tRNA synthetases from *E. coli* and yeast cytoplasm and mitochondria at a concentration of 0.5 mM. The modification under these conditions leads to a nearly parallel inhibition of enzymatic activities of only both hen liver enzymes to a plateau level of 15–20% after 10 min (not shown). A slight decrease of 5–10% in the ablity to hydrolyze preformed aminoacylated tRNA^{Phe} is noticed for both hen liver and yeast enzymes.

The inhibition due to specific modification of lysine residues

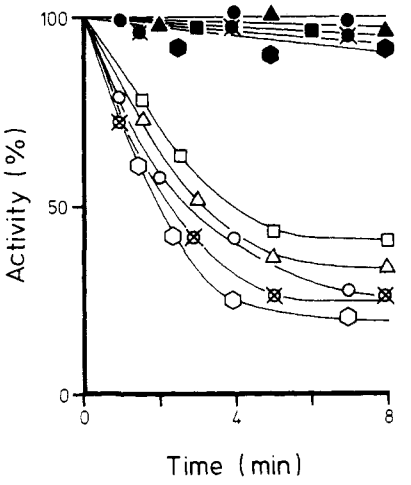

FIGURE 3: Influence of pyridoxal 5′-phosphate on aminoacylation activity of different phenylalanyl-tRNA synthetases. The open symbols correspond to experiments in the absence of tRNA; the full symbols, to the presence of 5 μM $tRNA^{Phe}$. A 5-fold excess of reagent, given as molar ratio to total lysine content of the enzymes, was added at 25 °C to absolute protein concentrations of 100–220 μg/mL and incubated for indicated periods of time before assaying. Phenylalanyl-tRNA synthetase from hen liver cytoplasm (crossed open circle, crossed solid circle), hen liver mitochondria (○, ●), yeast cytoplasm (△, ▲), yeast mitochondria (□, ■), and *E. coli* (○, ●).

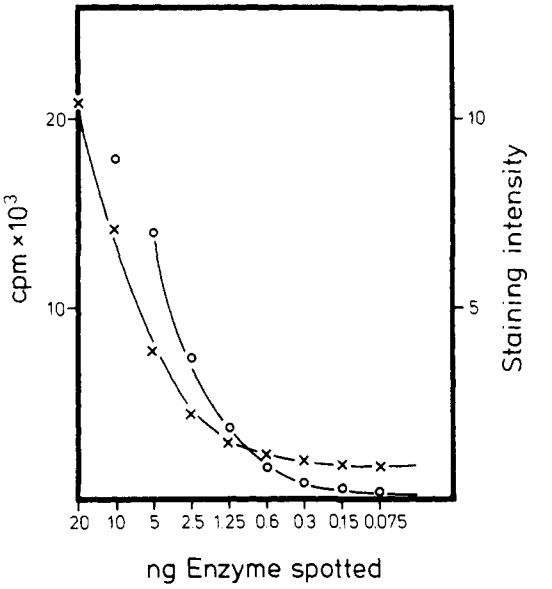

FIGURE 4: Comparison of sensitivities of antigen detection by protein A–peroxidase monoconjugate (○) or ^{125}I-labeled protein A (×). Staining intensity after 10 min of staining is expressed in the arbitrary units obtained from the densitometer integrator. In both cases, 1 μL of a 2-fold dilution of yeast or hen liver phenylalanyl-tRNA synthetase ranging from 20 to 0.075 μg was spotted on nitrocellulose.

was investigated by using pyridoxal 5′-phosphate. Under the described conditions, the ATP/PP_i pyrophosphate exchange activity was not affected, which is in accordance to earlier reports for the *E. coli* and yeast cytoplasmic phenylalanyl-tRNA synthetases (Baltzinger et al., 1979; Gorshkova et al., 1980). A similar inhibition mode of aminoacylation activity is seen for all enzymes, and the presence of 5 μM $tRNA^{Phe}$ during modification completely protects the aminoacylation activity (Figure 3). With $tRNA^{Phe}$, the AMP production also remains unaffected. The number of lysine residues modified, a molar extinction coefficient of 1.015×10^4 M^{-1} cm^{-1} at 325 nm (Piszkiewicz et al., 1977) being used, after a 50% decrease in the activity is eight to ten for the yeast enzymes and four to five for the other enzymes. The spectrophotometric determination was confirmed by amino acid analyses of modified enzymes. Thus, it can be assumed that the tested phenylalanyl-tRNA synthetases contain lysine residues involved in the recognition, positioning, and aminoacylation of $tRNA^{Phe}$ but not essential for activation of phenylalanine. The presence of a lysyl residue at the vicinity of the acceptor end of $tRNA^{Phe}$ was demonstrated recently for the yeast cytoplasmic phenylalanyl-tRNA synthetase (Renaud et al., 1982) and may be an important common property of aminoacyl-tRNA synthetases with the *E. coli* isoleucyl-tRNA synthetase as a further example (Piszkiewicz et al., 1977).

Immunological Characterization of Relationship. Immunological reagents provide versatile and sensitive tools for the elucidation of similarities and differences within enzymes of similar function from different species or intracellular compartments. To elucidate apparent evolutionary relatedness, we raised antibodies against the five phenylalanyl-tRNA synthetases in rabbits, applied standard immunological methods, and developed a highly sensitive modified immunospotting procedure using protein A–peroxidase monoconjugate to overcome the drawbacks of expense and short half-life of ^{125}I-labeled protein A. In comparison of sensitivity, the response of the two assays with protein A–peroxidase and ^{125}I-labeled protein A was linear within the same concentration range under the described conditions, although the monoconjugate assay was somewhat more sensitive (Figure 4). As can be seen, as little as 250 pg of antigen is readily detected in this system.

With serial dilutions of different tissue extracts, the sensitivity and reliability of the monoconjugate detection method were furthermore found comparable to the radioimmunoassay procedure with respect to the determination of concentrations of cytoplasmic phenylalanyl-tRNA synthetase in different organs of hen (not shown). Since the competition curves of all extracts tested proved superimposable with the standard curve, problems of interfering components can be excluded.

Ouchterlony immunodiffusion, immunoelectrophoresis, and immunoinactivation reveal cross-reactivity only for the intracellular heterotopic isoenzymes. Controls in immunoinactivation, consisting of the addition of IgG fractions from nonimmunized rabbits, generally showed no inhibition. A relatively higher sensitivity and information on the immunological relationship of the subunits are provided by Western blot immunodetection. Both subunits again of only the heterotopic intracellular isoenzymes cross-react (not shown). Since the phenylalanyl-tRNA synthetases comprise 10 ± 2 antigenic determinants with homologous F_{ab} fragments, both subunits therefore elicit immunological response in rabbits. It should be mentioned that this value corresponds to the minimum number of antigenic determinants because the binding of F_{ab} fragments may be hindered by steric factors.

The quantitation of the extent of immunological relationship by the highly sensitive immunospotting substantiates these results (Table IV). It is noteworthy that antibodies against mitochondrial phenylalanyl-tRNA synthetases from yeast and hen liver show no cross-reaction to cytoplasmic enzymes from closely related species, e.g., *Neurospora crassa* or turkey. Apart from steric hindrance of IgG molecules on the enzyme surface, this highly sensitive test unambiguously proves the absence of relationship between mitochondrial and the *E. coli* phenylalanyl-tRNA synthetases. This could not completely be excluded from immunoinactivation data, because enzymes may retain their activity upon binding of antibodies (Arnon, 1973). As a further control, it is shown that antibodies against yeast phenylalanyl-tRNA synthetases do not cross-react to

Table IV: Extent of Cross-Reaction (First Line) of Phenylalanyl-tRNA Synthetases from *E. coli* (Ec), Yeast Cytoplasm (Scc), Yeast Mitochondria (Scm), Hen Liver Cytoplasm (hlc), and Hen Liver Mitochondria (hlm) in the Heterologous Reaction as Percent of the Homologous Reaction (=100%) and $S\Delta Q$ Values (Second Line), Evaluated from Amino Acid Compositions

source of enzyme		source of antibody				
		Ec	Scc	Scm	hlc	hlm
Ec	cross-reaction	100	0	<1	0	4
	$S\Delta Q$ value		83	50	34	30
Scc	cross-reaction	<1	100	58	0	0
	$S\Delta Q$ value	83		10	21	23
Scm	cross-reaction	<1	61	100	0	3
	$S\Delta Q$ value	50	10		18	11
hlc	cross-reaction	0	0	0	100	34
	$S\Delta Q$ value	34	21	18		5
hlm	cross-reaction	0	0	2	26	100
	$S\Delta Q$ value	30	23	11	5	

yeast cytoplasmic valyl- and tyrosyl-tRNA synthetases. These different aminoacyl-tRNA synthetases therefore have no common antigenic determinants when our antibody preparations from rabbit and the highly sensitive immunospotting procedure are used.

The immunological approach to estimate the degree of structural resemblance can be compared to the estimation of relationship from differences in amino acid compositions according to the method of Marchalonis & Weltman (1971). The individual differences in mol % of the amino acids are squared and summed, yielding the so-called $S\Delta Q$ value. Marchalonis & Weltman found that within protein families the $S\Delta Q$ value correlates well with phylogenetic divergence and extent of amino acid sequence dissimilarity. If these calculations are done for the five enzyme species (Table IV), the range of $S\Delta Q$ values evidences consistent with the results of the immunospotting quantitation for evolutionary relationship of cytoplasmic and mitochondrial phenylalanyl-tRNA synthetases in lower and higher eukaryotic organisms.

As a probe for the structural relatedness of phenylalanyl-tRNA synthetase in different organisms, the immunological reactivity with immunoglobulins against cytoplasmic hen liver phenylalanyl-tRNA synthetase was tested. The results revealed considerable structural heterogeneity of this enzyme in the different vertebrate classes (Table V). The structural heterogeneity and the specificity of the method is evidenced by the lack of antibody-binding response in the case of other eukaryotic phenylalanyl-tRNA synthetases, e.g., from insects or nematodes. Also antiserum against both yeast and the *E. coli* phenylalanyl-tRNA synthetases showed no detectable cross-reactivity to enzyme preparations of all tested animal species. Although all these enzymes have an identical subunit structure, structural features thus can differ for enzymes catalyzing the same reaction in different organisms.

Table V: Extent of Cross-Reaction of Various Animal Cytoplasmic Phenylalanyl-tRNA Synthetases with Antibodies to the Hen Liver Cytoplasmic Enzyme Expressed as Percentage of the Homologous Reaction

source of enzyme	extent of cross-reaction
birds	
hen (*Gallus gallus*)	100
turkey (*Meleagris galloparo*)	82
pigeon (*Columba livia*)	67
duck (*Anas platyrhynchos*)	64
goose (*Anser anser*)	56
reptiles	
turtle (*Chelonia mydas*)	19
amphibia	
frog (*Rana ridibunda*)	14
artiodactyls	
ox (*Bos taurus*)	12
pig (*Sus scrofa*)	10
species of other mammalian orders	
cat (*Felis silvestris*)	10
horse (*Equus caballus*)	8
fishes	
carp (*Cyprinus carpio*)	6
trout (*Salmo fario*)	5
nonvertebrate species	
Drosophila melanogaster	
Caenorhabditis elegans	
ascomycetes	
Saccharomyces cerevisiae	

Table VI: Similarity in Comparison of Phenylalanyl-tRNA Synthetase Pairs

	ct/mt	ct/pk	mt/pk
amino acid composition ($S\Delta Q$)	+	−	−
trypsin pattern[b]	+	−	−
Ouchterlony double diffusion	+	−	−
immunoelectrophoresis	+	−	−
immunotitration	+	−	−
immunoblotting	+	−	−
immunospotting	+	−	−
active site mapping of ATP[c]	+	(+)	(+)
active site mapping of Phe	+	(+)	(+)
catalytic cooperativity[b]	+	+	+
active site labeling[b]	+	+	+
proofreading mechanism	+	−	−
chemical modification	+	−	−
glycoprotein[d]	−	+	−

[a] (+) High degree of similarity; ((+)) low degree of similarity; (−) no similarity. [b] Gabius & Cramer (1982b). [c] Gabius et al. (1982b). [d] Gabius et al. (1983c).

Discussion

The present functional and immunological study indicates a close relatedness between the intracellular heterotopic isoenzymes of phenylalanyl-tRNA synthetases in yeast and hen liver. The subtle differences at the amino acid binding site exploited by phenylalanine analogues between the enzyme pairs reflect variations in the structures of these distinct enzymes. Since the correct aminoacylation of tRNA with the proper amino acid by aminoacyl-tRNA synthetases is one of the key reactions, this step strongly contributes to the overall fidelity of protein biosynthesis (Cramer et al., 1979; Yarus, 1979). The accuracy of mitochondrial phenylalanyl-tRNA synthetases appears to be even better than the accuracy of their cytoplasmic counterparts. This is remarkable, as mitochondria only synthesize a limited set of hydrophobic proteins (Gray & Doolittle, 1982). The strategies to achieve this level of accuracy closely resemble those of the corresponding cytoplasmic enzymes, namely, pretransfer for the hen liver enzymes and posttransfer for the yeast enzymes and to a considerably smaller extent for the *E. coli* enzyme. We had suggested in a preceding paper (Gabius et al., 1983a) that the interplay between the active site of the enzyme, the 3′-terminal adenosine of tRNA^{Phe}, and the aminoacyl adenylate may give an intermediary complex that potentially can dissipate its energy into either pathway of proofreading. Other functional properties such as catalytic cooperativity and locations of the active site appear to be constant throughout evolution, when the results of comparative studies for phenylalanyl-tRNA synthetases are summarized (Table VI). Structural and immunological properties are only similar to the pair of heterotopic intracellular isoenzymes of one species. The only

exception from the closer resemblance of cytoplasmic and mitochondrial than cytoplasmic or mitochondrial and prokaryotic enzymes is the association of carbohydrates with mitochondrial phenylalanyl-tRNA synthetases (Gabius et al., 1983c). The functional role of carbohydrates that are also found with *Euglena gracilis* chloroplastic leucyl-tRNA synthetase (Imbault et al., 1981) and cytoplasmic rat liver arginyl- and lysyl-tRNA synthetases (Dang et al., 1982) is unknown.

The results allow one to decide between the three possible events to explain the cytoplasm–mitochondrion isoenzyme pair: (1) duplication of a eukaryotic gene and subsequent incorporation of one of the two enzymes into the organelle; (2) gene transfer from endosymbiont; (3) duplication of transferred gene and incorporation of one enzyme into the organelle, the other becoming functional in the cytoplasm. From the presented data summarized in Table VI, we conclude that the ancestral gene was in all probability eukaryotic. The different genes in the nuclear DNA thus would have arisen by gene duplication. The close intraspecies resemblance suggests independent duplication after the separation of the evolutionary branches of fungi and animals. Other examples of similarity for aminoacyl-tRNA synthetases of different intracellular components, as judged by common antigenic determinants in serological tests, include the mitochondrial and cytoplasmic valyl-tRNA synthetases from yeast and *Tetrahymena pyriformis* (Suyama & Hamada, 1978; Felter et al., 1981) but not the *Tetrahymena* leucyl-tRNA synthetases (Chiu & Suyama, 1973). Our data argue against the origin of this enzyme pair by gene transfer from the endosymbiont as pointed out for the animal superoxide dismutases (Steinman & Hill, 1973) or a large number of nuclear encoded chloroplast genes (Weeden, 1981). Mitochondrial $tRNA^{Phe}$, on the other hand, bear no distinctive resemblance either to the known prokaryotic or to eukaryotic cytoplasmic $tRNA^{Phe}$ (Hasegawa et al., 1981). As mitochondria and chloroplasts have a number of common features, it is noteworthy that with respect to tRNA sequences and structural properties of valyl- and leucyl-tRNA synthetases, a higher degree of homology between chloroplastic and bluegreen algal tRNA and chloroplastic and prokaryotic enzymes is reached than in relation to their cytoplasmic counterparts (Imbault et al., 1981; Colas et al., 1982; Gauss & Sprinzl, 1983). The immunological comparison revealed considerable structural heterogeneity among the different phenylalanyl-tRNA synthetases. The degree of structural homology between phenylalanyl-tRNA synthetases from higher and lower eukaryotic organisms is not sufficient to generate common antigenic determinants and, even within the class of vertebrates, antigenic determinants do not appear to be very conservative. Although it is likely that conserved regions are involved in maintaining the structure and function of the enzyme and these are likely to be located to some extent in the interior of the protein, the situation is different to other enzymes in nucleic acid biochemistry. Structural relatedness for enzymes from phylogenetically very different organisms was noted for the large polypeptides of eukaryotic RNA polymerase β (Huet et al., 1982), the DNA polymerase β (Chang et al., 1982), and the high molecular weight replication DNA polymerase (Hübscher et al., 1981). It can therefore be concluded that phenylalanyl-tRNA synthetases do not belong to the group of highly conserved enzymes in gene expression.

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Registry No. Phe, 63-91-2; Tyr, 60-18-4; Leu, 61-90-5; Met, 63-68-3; *p*-fluorophenylalanine, 1132-68-9; β-phenylserine, 2584-75-0; 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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Critical Lysine Residue at the Chloride Binding Site of Angiotensin Converting Enzyme†

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ABSTRACT: Pulmonary angiotensin converting enzyme has been reductively methylated by using formaldehyde and sodium cyanoborohydride. This modification virtually eliminates enzyme activity toward some substrates (e.g., furanacryloyl-Phe-Gly-Gly) while less drastically affecting activity toward others (e.g., furanacryloyl-Phe-Phe-Arg). Affinity chromatography and analysis of radiolabeled reaction products reveal that this effect is due to methylation of a single critical lysine residue. Loss of activity primarily represents an increase in K_m values, indicating that the critical lysine plays a role in substrate binding. This lysine can be protected by a competitive inhibitor, suggesting that it is at or near the active site. Addition of chloride at pH 6.1 specifically protects against methylation of this lysine. These findings support the idea that the critical lysine is part of the binding site for chloride and other monovalent anions which are strong activators of the enzyme.

Angiotensin converting enzyme (dipeptidyl carboxypeptidase, EC 3.4.15.1) (ACE)[1] is a zinc metalloexopeptidase that releases dipeptides from the C-terminus of a wide variety of oligopeptide substrates (Soffer, 1976). Chemical modification studies from this laboratory (Bünning et al., 1978) and others (Fernley, 1977; Harris & Wilson, 1982, 1983) have suggested that the active site of ACE is closely related to that of carboxypeptidase A (Vallee et al., 1983; Lipscomb et al.,

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[1] Abbreviations: ACE, angiotensin converting enzyme; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; FA, *N*-furanacryloyl; *t*-Boc, *tert*-butyloxycarbonyl; PPPP, *N*-(phenylphosphoryl)-L-Phe-L-Phe; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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