Biochemistry

 Volume 20, Number 25 December 8, 1981

Discrimination between D- and L-Tyrosyl Transfer Ribonucleic Acids in Peptide Chain Elongation†

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ABSTRACT: D-Tyr-tRNA can take part in peptide bond formation with *N*-AcPhe-tRNA on ribosomes programmed with the hexanucleotide UUUUAC. None of the steps leading to peptide bond formation exhibit high stereoselectivity. Ternary complex formation with EF-Tu·GTP favors L-Tyr-tRNA by a factor greater than 25. The complex formed with D-Tyr-tRNA was not protected from hydrolysis, which suggests that the D-amino acid is improperly bound to the protein. The rate of EF-Tu-promoted dipeptide formation was 30-fold faster with L-Tyr-tRNA. The ratio of moles of GTP hydrolyzed to dipeptide formed was 1.4 for L-Tyr-tRNA and 4 for D-Tyr-tRNA. The excess of GTP hydrolyzed to peptide bonds formed is evidence for kinetic proofreading in AA-tRNA selection. The combined effects of the partial discrimination at each stage, from the aminoacylation to the peptide formation, favor L-tyrosine by a factor greater than 10^4 and would virtually exclude D-tyrosine from being incorporated under conditions where L-tyrosine was also present.

In 1967, Calendar and Berg reported the interesting observation that D-tyrosine could be esterified to $tRNA^{Tyr}$ to the same extent as L-tyrosine and then could be incorporated into peptides, at about one-sixth the rate of the natural enantiomer, in an in vitro protein-synthesizing system consisting of ribosomes, poly(U,A) (3:1), and an S-100 fraction (Calendar & Berg, 1966a,b, 1967). In the succeeding years, the details of peptide chain elongation have been elucidated, and at present, the mechanism of aminoacyl-tRNA (AA-tRNA)[1] selection is being studied extensively. We decided to reexamine the reactions leading to incorporation of D-tyrosine into peptides in order to determine the enantiomeric selectivity of each step of the process and to determine whether discrimination mechanisms such as kinetic proofreading limit the incorporation of D-amino acids. A previous investigation showed that D-tyrosine is esterified to $tRNA^{Tyr}$ more slowly than is L-tyrosine. Because only 1 mol of ATP is consumed during the acylation (Yamane & Hopfield, 1977), the preferential selection of L-tryosine must be determined by steric interactions rather than by kinetic proofreading. Using $tRNA^{Tyr}$ selectively aminoacylated with D- or L-tyrosine, we have investigated the affinities of EF-Tu·GTP for the enantiomeric AA-tRNAs, have compared the binding of the D- and L-Tyr-tRNA·EF-Tu·GTP complexes to ribosomes, and have examined the ratios of GTP hydrolysis to peptide bond formation directed by the synthetic template UUUUAC.

Materials and Methods

Ultrogel AcA-44 is a product of LKB, Inc. Sephadex G-50 was obtained from Pharmacia. Purified $tRNA^{Phe}$ and $tRNA^{Tyr}$ from *Escherichia coli* MRE 600 were from Boehringer-Mannheim and had an acceptance activity of 1200 and 1010 pmol/A_{260} unit, respectively. The tRNAs were charged with the purified amino acids (see below) as previously described. *N*-AcPhe-tRNA was prepared by the method of Haenni & Chapeville (1966). Ribosomes were isolated from *E. coli* MRE 600 and washed in a buffered salt solution containing 1 M NH_4Cl, as described (Brot et al., 1971). Elongation factors Tu and G, initiation factor mixture, and AA-tRNA synthetases were prepared by published procedures (Miller & Weissbach, 1974; Brot et al., 1971; Littauer, 1971). L-Amino-acid oxidase [L-amino-acid:oxygen oxidoreductase (deaminating), EC 1.4.3.2] and catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase, EC 1.11.1.6) were obtained from Worthington Biochemical Corp. Nonradioactive L- and D-amino acids were purchased from Calbiochem and were purified as described below. Radioactive amino acids, L-[^{14}C]tyrosine, [^{3}H]phenylalanine, and D-[^{3}H]tyrosine, and [γ-^{32}P]GTP were obtained from New England Nuclear Corp.

Purification of Amino Acids. D-Tyrosine (10 nmol, 1.1×10^5 cpm/pmol) was treated with a sequential addition of 1 unit of L-amino-acid oxidase every 30 min, in a total of 5 units, and 1 unit of catalase in 1 mL of 50 mM Tris-HCl (pH 7.8) for 3 h at 37 °C. The reaction was stopped by immersing the reaction tube in boiling water for 2 min, acidified by the addition of 1 mL of 400 mM acetic acid, and centrifuged. The

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[1] Abbreviations used: AA-tRNA, aminoacyl-tRNA; EF-Tu, elongation factor Tu; DTT, dithiothreitol.

0006-2960/81/0420-7059$01.25/0

supernatant was passed through a 0.75 × 5 cm column of Dowex 50-H^+, and *p*-hydroxyphenyl pyruvate was washed off with 200 mM acetic acid (5 × 2 mL). D-Tyrosine was eluted with 6 N HCl (Calendar & Berg, 1967). This treatment was twice repeated, and the amino acid was then subjected to thin-layer chromatography on DEAE-cellulose (solvent: 1-butanol–acetic acid–water, 18:2:5 v/v) to remove impurities which travelled with the solvent front and close to the dipeptide *N*-AcPhe-Tyr.

Preparation of Oligonucleotide Template. The hexanucleotide UUUUAC was prepared by starting with $UUUU_{OH}$ (Boehringer) and by stepwise addition of pAp and pCp by RNA ligase as described previously (Uhlenbeck & Cameron, 1977; England et al., 1977). The intermediate product, $UUUUA_p$, was treated with bacterial alkaline phosphatase to remove the 3′-terminal phosphate before the addition of pCp. The product of each step was isolated by 3 MM paper chromatography followed by gel filtration on Bio-Gel P-2 (Bio-Rad). This preparation was carried out in Dr. O. C. Uhlenbeck's laboratory.

Assay Procedures. The interaction of L- or D-Tyr-tRNA with EF-Tu·GTP was investigated by three procedures. In the nitrocellulose filtration assay, the ternary complex AA-tRNA·EF-Tu·GTP passes through the filter, whereas EF-Tu·GTP is bound to the filter. In the protection from hydrolysis assay, AA-tRNA bound to EF-Tu·GTP resists hydrolysis, whereas free AA-tRNA is rapidly deacylated. In the gel filtration procedure, the ternary complex emerges from the column ahead of EF-Tu·GTP or AA-tRNA. Details of the experimental conditions are given in the figure legends. The binding of AA-tRNA to ribosomes was measured by the method of Nirenberg & Leder (1964). Dipeptide synthesis was estimated by hydrolysis of the reaction mixture at 37 °C for 30 min in 0.1 M KOH. The mixture was then acidified to pH 1 and extracted in ethyl acetate as described (Erbe & Leder, 1968; Erbe et al., 1969). The extract was evaporated to dryness, taken up in 0.1 mL of ethyl acetate, applied to TLC–DEAE–cellulose, and developed in the previously described solvent. Segments of the chromatogram were taken for liquid scintillation counting, and the migration rates of the labeled peptides were compared to those of authentic samples of *N*-AcPhe-Tyr (R_f = 0.93), *N*-AcPhe-Phe (R_f = 0.98), *N*-AcPhe (R_f = 0.74), Phe (R_f = 0.53), and Tyr (R_f = 0.43). Hydrolysis of GTP was estimated by the acid molybdate extraction procedure (Modolell & Vanquez, 1973).

Results

Interaction of EF-Tu·GTP with L- *or* D-*Tyr-tRNA.* By use of the cellulose nitrate filter assay, a titration of EF-Tu·[^{3}H]GTP with L- or D-Tyr-tRNA revealed (Figure 1) that the species bearing L-tyrosine interacted with the protein–nucleotide complex much more strongly than the D-tyrosine-bearing derivative. The GTP concentration in the assay mixture (1×10^{-5} M) was sufficiently above the dissociation constant of EF-Tu·GTP (3×10^{-7} M) so that virtually all of the protein contained bound nucleotide; therefore, the results in Figure 1 can be used directly to calculate dissociation constants for the tRNAs from the ternary complexes. These data are consistent with $K_{diss} = (5 \pm 1) \times 10^{-8}$ M for the L-tyrosine-containing complex and $(12 \pm 2) \times 10^{-7}$ M for that containing D-tyrosine, in reasonable agreement with the value reported in Pingoud & Urbanke (1980) considering the semiquantitative nature of the present assay method. The cellulose nitrate filter method has the advantage of being fast and relatively sensitive, but the nature of the absorption is not understood, and it seems to depend on the amino acid.

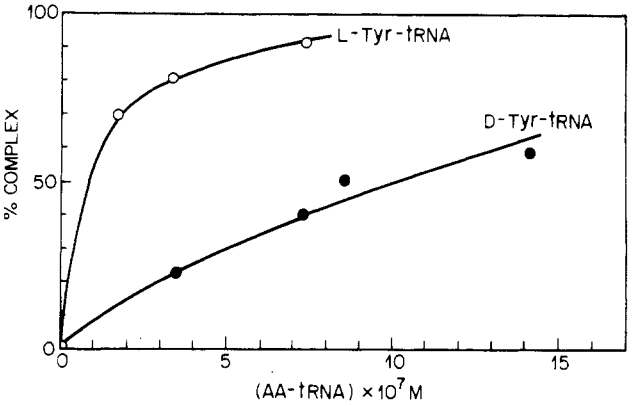

FIGURE 1: Titration of EF-Tu·GTP with L- and D-Tyr-tRNA by the nitrocellulose filtration assay. A 200-μL solution composed of 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NH_4Cl, 0.1 μM EF-Tu·[^{3}H]GTP (320 cpm/pmol), 10 μM GTP, and increasing amounts of L- or D-Tyr-tRNA as indicated was used. After 10 min at 0 °C, 5 mL of cold buffer was added and rapidly filtered through a nitrocellulose membrane (0.45-μm pore size) followed by washing with 3 × 5 mL of cold buffer. Since EF-Tu·GTP is absorbed to the nitrocellulose filter but the ternary complex is not, the difference in the amounts of filter-bound EF-Tu·GTP between the control and the sample containing L- or D-Tyr-tRNA represents the amount of ternary complex formed. Radioactivity was measured on a scintillation counter. (○) L-Tyr-tRNA; (●) D-Tyr-tRNA.

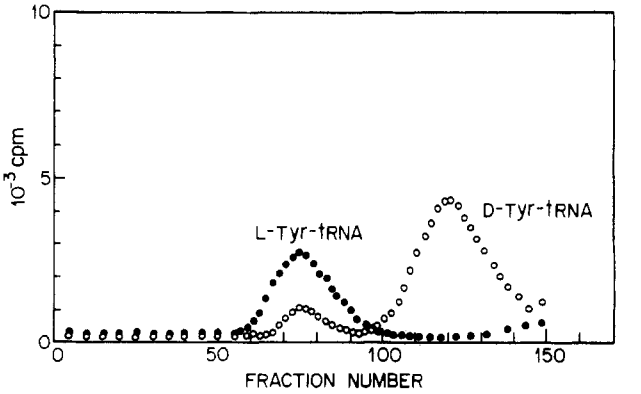

FIGURE 2: Gel chromatography of [^{3}H]D-Tyr-tRNA and [^{14}C]L-Tyr-tRNA and EF-Tu·GTP complexes on LKB Ultrogel AcA-44. Ternary complexes were prepared as previously described (Miller & Weissbach, 1974): [^{3}H]D-Tyr-tRNA, 3.3 μM, and EF-Tu·GTP, 42 μM; [^{14}C]L-Tyr-tRNA, 1.2 μM and EF-Tu·GTP, 3.5 μM. The column (1.5 × 25 cm) was equilibrated with a buffer containing 300 mM NaCl, 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 75 mM NH_4Cl, 75 mM KCl, 5 mM DTT, 5 μM GTP, and 0.1 μM EF-Tu·GTP. Fractions of 10 drops (0.55 mL) each were collected and counted on a scintillation counter. (●) L-Tyr-tRNA; (○) D-Tyr-tRNA.

Interaction between EF-Tu·GTP and D-Tyr-tRNA was also demonstrated by gel filtration chromatography (Figure 2). The columns were equilibrated beforehand with EF-Tu·GTP to limit dissociation of the labile complexes, a procedure that enabled us to observe reproducibly an interaction with D-Tyr-tRNA, whereas in the usual method without ligand equilibration the complex dissociated. The column behavior of the D-Tyr-tRNA-containing complex confirms that is much less strongly associated than the L-Tyr-tRNA·EF-Tu·GTP complex. Even with a large excess of EF-Tu·GTP, only a minor fraction of D-Tyr-tRNA eluted ahead of free tRNA, whereas with a moderate excess of EF-Tu·GTP, all of the L-Tyr-tRNA appears in the rapidly eluted ternary complex.

To be certain that the apparent reactivity of the D-Tyr-tRNA was not produced by a small contamination by the L isomer, we tested the slowly emerging peaks of free D-Tyr-tRNA for reactivity in the preceding filter assay. As the results of Table I show, the D-Tyr-tRNA purified by the gel

Table I: Ternary Complex Formation of Purified D-Tyr-tRNA^{Tyr} with EF-Tu·GTP Determined by the Nitrocellulose Filter Assay Method[a]

expt	additions (pmol)	EF-Tu·GTP retained on filters (pmol)
1	none	16.5
2	L-Tyr-tRNA (20)	3.2
3	purified D-Tyr-tRNA (25)	13.7
4	purified D-Tyr-tRNA (50)	11.8
5	L-amino-oxidase-treated D-Tyr-tRNA (21)	11.5
6	untreated D-Tyr-tRNA (21)	13.5
7	nonacylated tRNA^{Tyr} (55)	15.1

[a] Purified D-Tyr-tRNA^{Tyr} was prepared from the first peak in Figure 2 by ethanol precipitation. Untreated refers to D-Tyr-tRNA^{Tyr} before the column fractionation treatment. Nonacylated tRNA^{Tyr} refers to commercial (Boehringer) tRNA^{Tyr} dissolved in 0.1 M NH_4OH, pH 10, incubated at 37 °C for 20 min and ethanol precipitated.

filtration procedures had the same low but measurable reactivity as the unpurified D-Tyr-tRNA and tRNA charged with enzymatically purified D-tyrosine.

Binding to EF-Tu·GTP is known to greatly diminish the rate of deacylation of the L-aminoacyl-tRNAs (Beres & Lucas-Lenard, 1973). Under our experimental conditions, with a 3-fold excess of EF-Tu·GTP, L-Tyr-tRNA is almost completely protected throughout the interval during which unbound L- or D-Tyr-tRNA is deacylated to an extent of 95% (Figure 3). In contrast, D-Tyr-tRNA is only slightly protected by a 20-fold excess of EF-Tu·GTP. The dissociation constant of D-Tyr-tRNA from the ternary complex (K_{diss} = 1.2 μM) predicts that, at the concentration employed (2.1 μM D-Tyr-tRNA, 42 μM EF-Tu·GTP), less than 3% of D-Tyr-tRNA is dissociated from EF-Tu·GTP. However, there is a possibility that the K_{diss} value determined by the present cellulose nitrate filter assay method is lower than it actually is. The observed high rate of deacylation of D-Tyr-tRNA indicates that ternary complex formation does not protect this aminoacyl bond from hydrolysis, suggesting that the D-tyrosyl residue is highly exposed to solvent.

Binding of D- *and* L-*Tyr-tRNAs to Ribosomes.* In order to study the codon-directed binding of D- and L-Tyr-tRNAs to ribosomes and the subsequent incorporation of the amino

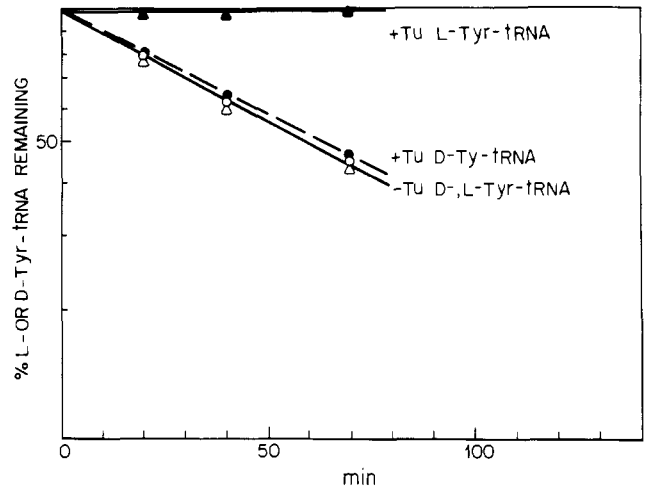

FIGURE 3: Effect of EF-Tu·GTP upon the rates of deacylation of L- and D-Tyr-tRNA. The reactions were conducted at 37 °C in 500 μL containing 50 mM Tris-HCl (pH 7.0), 10 mM $MgCl_2$, 50 mM NH_4Cl, and 1.2 μM L-Tyr-tRNA + 3.5 μM EF-Tu·GTP or 2.2 μM D-Tyr-tRNA + 42 μM EF-Tu·GTP. Samples (100 μL) were taken at the time intervals indicated, and trichloroacetic acid was precipitated and counted on a scintillation counter. (○) D-Tyr-tRNA, −EF-Tu·GTP; (●) D-Tyr-tRNA, +EF-Tu·GTP; (△) L-Tyr-tRNA, −EF-Tu·GTP; (▲) L-Tyr-tRNA, +EF-Tu·GTP.

acids into dipeptides, we used the hexanucleotide message UUUUAC, coding for Phe-Tyr. With or without added initiation factors, *N*-AcPhe-tRNA bound 27–40% as efficiently in response to the hexanucleotide template as to poly(U) (Table II). L-Tyr-tRNA bound to these preinitiated ribosomes nearly 3 times more efficiently in the presence of EF-Tu·GTP than in its absence at 0 °C and 10-min incubation. The stoichiometric ratio of bound L-Tyr-tRNA to *N*-AcPhe-tRNA may be calculated to be 0.7 or 1.0, depending upon whether one chooses to correct for the template-independent binding. The D-Tyr-tRNA binding data (Table II) suffer from rather low picomoles bound values, but taking into account experimental uncertainties, one can estimate that in the presence of EF-Tu·GTP, D-Tyr-tRNA binds to ribosomes approximately 30% as efficiently as L-Tyr-tRNA does, namely, 0.25 pmol/A_{260} unit of ribosomes for the D isomer compared to 0.75 pmol/A_{260} unit or ribosomes for the L isomer (Table II). The

Table II: Effect of Initiation and Transfer Factors (IF, Tu·GTP, and G) on *N*-AcPhe-tRNA and Tyr-tRNA Binding to Ribosomes at 10 mM Mg^{2+} in Response to UUUUAC[a]

expt	mRNA	IF	EF-G	EF-Tu·GTP (μM)	*N*-AcPhe (μM)	L-Tyr bound (pmol)	D-Tyr
1	U_4AC	+	−	−	0.33	−	−
2	+	−	−	−	0.36	−	−
3	U_6	+	−	−	0.32	−	−
4	poly(U)	+	−	−	0.89	−	−
						σ	σ
5	U_4AC		+	2.4		0.29 (0.01)	0.09 (0.01)
6	+		−	2.4		0.29 (0.01)	0.10 (0.01)
7	+		−	12		0.30 (0.01)	0.15 (0.02)
8	+		−	−		0.10 (0.02)	0.08 (0.02)
	poly(UAC) (1:1:1)						
9	30 S		−	−		1.51	1.64
10	70 S		−	−		1.15	0.43

[a] Average of five experiments. The 50-μL reaction volume contained 50 mM Tris-HCl (pH 7.4), 10 mM $Mg(OAc)_2$, 50 mM NH_4Cl, 5 mM DTT, 2 μg of IF, 10 μg of G factor, EF-Tu·GTP, 66 pmol of *N*-AcPhe-tRNA (1960 cpm/pmol), 64 pmol of [^{14}C]L-Tyr-tRNA (1700 cpm/pmol), 65 pmol of [^{3}H]D-Tyr-tRNA (10 900 cpm/pmol), 0.4 A_{260} unit of ribosomes, 0.1 A_{260} unit of U_4Ac, U_6, and poly(U), and 1 mM GTP. Incubation was carried out at 0 °C for 10 min and assayed as described by Nirenberg and Leder. In the first four experiments, no D- or L-Tyr-tRNA was added. In experiments 5–8, D- and L-Tyr-tRNAs were added separately to identical mixtures containing unlabeled *N*-AcPhe-tRNA. Experiments 9 and 10 were carried out according to Miskin et al. (1970) and Zamir et al. (1971, 1974) in the presence of 0.3 A_{260} unit of 30S or 70S ribosomes, 0.2 A_{260} unit of poly(UAC), and 11 pmol of L-Tyr-tRNA or 12 pmol of D-Tyr-tRNA. Control (−mRNA) values have been subtracted for calculations. σ = standard deviation.

Table III: Concentrations of Components in the Dipeptide Reaction[a]

	free tRNA (μM)	free EF-Tu or GTP (μM)	EF-Tu·GTP (μM)	ternary complex (μM)
D-Tyr-tRNA	0.26	0.32	0.34	0.07
L-Tyr-tRNA	0.06	0.27	0.24	0.24

[a] The concentration of each species was calculated for the mixtures described in Figure 4 by using the dissociation constants quoted in the text.

extent of binding is limited by the amount of correctly bound hexanucleotide template. Certainly, the yield could have been higher with AUGUAC as the mRNA, but because of its base complementarity, this hexanucleotide has the possibility of forming a dimer and consequently lowering its efficiency as mRNA. The binding efficiency is better in the presence of poly(AUC) (1:1:1) (Table II). As expected, no difference between L- and D-Tyr-tRNA binding to 30S subunits was observed.

Rates of GTP Hydrolysis and D- *or* L-*Tyr-tRNA Binding.* To determine where in the sequence of interactions leading to dipeptide formation the discrimination occurs, we examined the relationship between the rates of dipeptide formation and hydrolysis of GTP in the ternary complex. The time courses of GTP hydrolysis and dipeptide formation for D-Tyr-tRNA- and L-Tyr-tRNA-containing ternary complexes differ greatly (Figure 4). The synthesis of the L-Tyr dipeptide is accelerated by about a factor of 7 by added EF-Tu·GTP, whereas D-Tyr dipeptide formation is accelerated less than 5% by the enzyme. It is a small effect but real, and yet is sine qua non for the argument of the paper. The greater stimulation of L-Tyr dipeptide formation is due in part to the greater stability of the L-Tyr-tRNA-containing ternary complex. The fraction of D-Tyr-tRNA in the ternary complex is low in these mixtures, because the [^{32}P]GTP concentration must be kept low to minimize the [^{32}P]PO_4^{3-} blank. Using the previously determined dissociation constant for EF-Tu·GTP ($K_{diss} = 3 \times 10^{-7}$ M) (Miller & Weissbach, 1970), we can calculate the amounts of free Tyr-tRNA and ternary complex (Table III). At least 80% of L-Tyr-tRNA remains in the ternary complex, whereas only 20% or less of D-Tyr-tRNA is so bound.

The amounts of GTP hydrolysis per peptide bond formed are 1.4 for L-Tyr-tRNA and 0.9 for D-Tyr-tRNA, respectively (Figure 4C,F). It is necessary to correct these ratios for the fraction of peptide bonds formed via the nonenzymatic (non-EF-Tu) pathway. For L-Tyr-tRNA, this calculation is straightforward. Because EF-Tu·GTP accelerates the rate of dipepitde formation by a factor of 7 (Figure 4), at least 85% of the reaction must proceed through the ternary complex. Furthermore, complexation with EF-Tu·GTP lowered the concentration of free L-Tyr-tRNA by a factor of 5, and it is reasonable to assume that the nonenzymatic rate of incorporation is consequently also lowered by a factor of 5.[2] Therefore, as much as 97% of L-tyrosine is incorporated via the EF-Tu pathway, and the ratio of GTP hydrolyzed to peptide bonds formed via this pathway is 1.4. This high value may in part result from the uncertainty in the high background correction.

[2] Although the assumption that the rate of dipeptide formation increases directly with Tyr-tRNA concentration in the range 0–0.3 μM has not been demonstrated, we have found, in experiments not shown, that the rate of binding of free L-Tyr-tRNA to ribosomes programmed with poly(UAC) increases directly with [L-Tyr-tRNA] in this concentration range, as the ribosomes are far from being saturated with Tyr-tRNA.

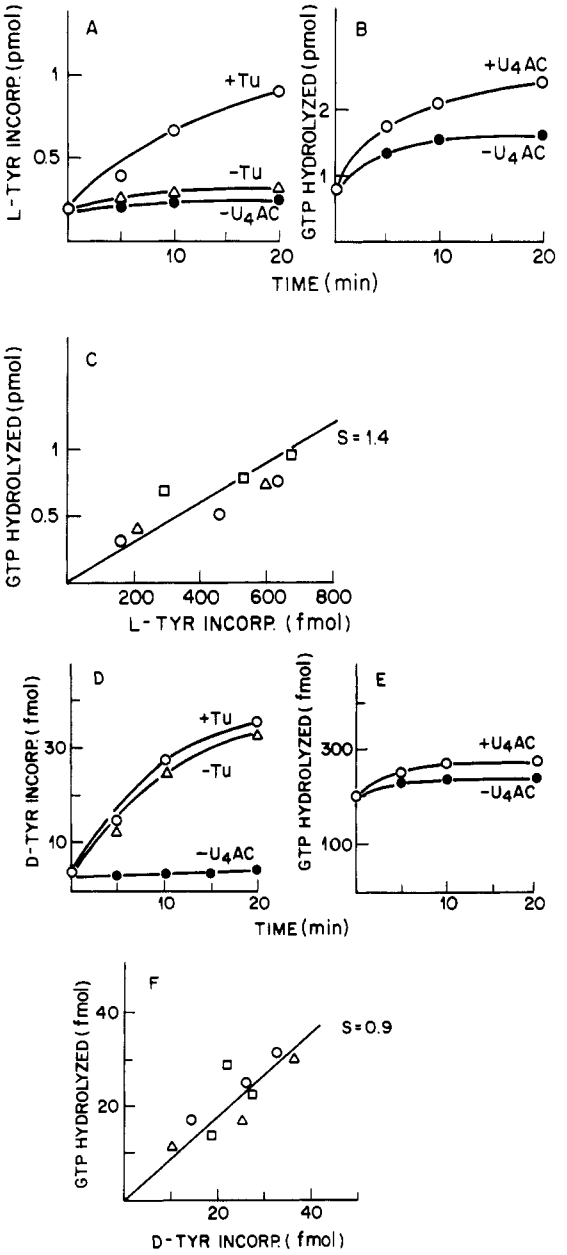

FIGURE 4: Rates of GTP hydrolysis and dipeptide formation following the interaction of the ternary complexes with ribosomes. Each complete reaction mixture (300 μL) contained 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 50 mM NH_4Cl, 2.4 A_{260} unit of ribosome, 0.6 A_{260} unit of UUUUAC, 160 pmol of *N*-AcPhe-tRNA, 98 pmol of [^{14}C]L-Tyr-tRNA (1700 cpm/pmol), 102 pmol of [^{3}H]D-Tyr-tRNA (10 900 cpm/pmol), and 224 pmol of EF-Tu·GTP (γ-^{32}P, 2400 cpm/pmol). Reactions were performed at 0 °C. (A, D) Kinetics of L-Tyr and D-Tyr incorporation and effect of EF-Tu·GTP: (○) +EF-Tu-GTP; (△) −EF-Tu·GTP; (●) −U_4AC. (B, E) GTP hydrolysis during L-Tyr and D-Tyr incorporation: (○) +U_4AC; (●) −U_4C. (C, F) Plot of GTP hydrolyzed per L-Tyr and D-Tyr incorporated: (○, △, □) three different sets of experiments. Samples (50 μL) were taken for dipeptide analysis, and 20-μL samples were used for GTP hydrolysis. Due to the isomerase contamination in the IF preparation, the reaction was carried out at 10 mM Mg^{2+}.

The incorporation of D-tyrosine into the dipeptide is only slightly accelerated by EF-Tu·GTP under the conditions of the assay (Figure 4D). As shown in Table III, since 80% of D-Tyr-tRNA remains uncomplexed, about 80% of the *N*-AcPhe-D-Tyr must still be formed from free D-Tyr-tRNA.[2] Therefore, only 20–25% of the total dipeptide is formed via the ternary complex, and consequently, the ratio of GTP hydrolyzed per peptide bond must be corrected by a factor of 4–5, becoming 3.6–4 instead of 0.9.

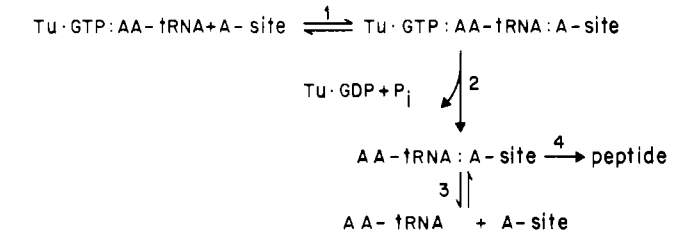

FIGURE 5: Schematic representation of AA-tRNA binding to the A site and peptide formation. There are two discrimination processes working at the stage of ternary complex binding to ribosomes, the first process occurring during the initial reversible binding of the complex to the ribosome (path 1), and the second one consisting of a proofreading step following the hydrolysis of GTP. This is an irreversible process (path 2). Once AA-tRNA is on the A site there is still a probability of being released (path 3) instead of being incorporated into a peptide chain (path 4), depending on the off rate of AA-tRNA bound. Nonenzymatic binding of AA-tRNA to the A site can occur through path 3.

Two assumptions used in this calculation may actually underestimate this ratio: (a) if the rate of dipeptide formation did not increase directly with free D-Tyr-tRNA, the amount of dipeptide formed by this pathway would be underestimated, and the GTP/total dipeptide ratio would increase; (b) if one uses the K_{diss} value quoted by Pingoud & Urbanke (1980), the ratio rises to a value greater than 10.

From these results, the stereoselectivity of each step of the binding reaction (Figure 5) can be estimated. Interaction of ternary complexes with ribosomes (reaction 1) must readily lead to GTP hydrolysis (reaction 2), because a stable association of ribosomes with ternary complexes containing GTP has not been observed. The overall rate of dipeptide formation,[3] in the presence of EF-Tu·GTP, can be calculated from Figure 4A,D, and one obtains a value of 0.91 for L-Tyr-tRNA and 0.029 pmol (10 min)$^{-1}$ (A_{260} unit of ribosomes)$^{-1}$ for D-Tyr-tRNA, a 30-fold rate difference between the isomers. Not all ternary complexes bound to ribosomes are successfully incorporated into peptide, and after GTP hydrolysis, a certain fraction of the AA-tRNA dissociates from ribosomes, via reaction 3, Figure 5. The fraction dissociated can be calculated from the ratio (rate of GTP hydrolysis − rate of peptide bond formation)/rate of GTP hydrolysis, and one can estimate that 75–90% of D-Tyr-tRNA dissociates from the ribosomes, whereas less than 30% of L-Tyr-tRNA is lost through this pathway. Let us define relative incorporation efficiency as being 1 − fraction dissociated, which is equal to the ratio K_4/K_2 in Figure 5. Then the ratio of relative incorporation efficiency between L-Tyr and D-Tyr becomes approximately 5, which may be attributed to kinetic proofreading and accounts for part of the 30-fold rate differences. The 6-fold rate difference yet remaining must occur in reactions 1 and 2.

Previous studies have examined the role of the anticodon and ribosomal interactions in AA-tRNA selection (Thompson & Stone, 1977; Yates, 1979; Campuzano et al., 1979). In these studies, in a specific mRNA-programmed ribosome system with noncognate aminoacyl-tRNAs, GTP hydrolysis uncoupled from stable binding has been observed, and this has been taken as positive evidence for the kinetic proofreading proposed by Hopfield (1974). We have found that the aminoacyl group also exerts a strong effect upon selection. In the case of tyrosine, the reactions—aminoacylation of tRNA, ternary complex formation with EF-Tu·GTP, ribosome binding, and peptide bond formation—show a certain degree of stereoselectivity but less than that commonly expected of enzymatic reactions. Several detailed studies on the stereospecificity of the peptidyltransferase reaction have been published (Nathans & Neidler, 1963; Rychlík et al., 1970; Harris et al., 1971; Ekerman et al., 1974; Bhuta et al., 1981), and results are somewhat contradictory depending on the system used. One reason is because as one tries to construct a simpler reaction system, relative to natural polypeptide synthesis, its specific requirements become more stringent. Another cause of the discrepancy might be due to a partial racemization of D-aminoacyl derivatives. A carefully executed experiment by Bhuta et al. (1981) has revealed that in the system *E. coli* fMet-tRNA·A-U-G·70S ribosomes the requirements for stereospecificity in peptidyl transfer reactions are not absolute, and the acceptor activity of the fragment C-A(L-Phe) is, at least, 50 times greater than that of the isomer, C-A(D-Phe). Our present data show that the rate of EF-Tu-promoted dipeptide formation is, approximately, 30-fold faster with L-Tyr-tRNA compared to D-Tyr-tRNA, in reasonably good agreement with the data of Bhuta et al. (1981).

Nevertheless, the combined effects of the partial selectivity at each stage would virtually exclude D-tyrosine from being incorporated under conditions where L-Tyrosine were also present. The maximal selectivity that might be realized would include factors of 25 for aminoacylation (Calendar & Berg, 1966a,b), 25 for ternary complex formation, 10 for EF-Tu·GTP-promoted binding, and 5 for peptidyl transfer, a total discrimination factor greater than 10^4. Despite this formamide barrier to incorporation of D-tyrosine from racemic mixtures, the partial selectivity of the pathway allows the incorporation of D-tyrosine at an appreciable rate if no L-tyrosine is present, as the work of Calendar and Berg has shown.

The sequence of events in the binding reaction appears to be the following: the ternary complex undergoes an initial screening based on the codon–anticodon interaction. Good-fitting complexes, therefore, with a long residence time, bind more extensively, which requires an interaction between the ribosome and the aminoacyl residue or a region of EF-Tu near the aminoacyl residue. Complexes unable to engage in this interaction would dissociate due to its fast "off rate", whereas the formation of this interaction would trigger GTP hydrolysis. At this stage, the bound AA-tRNAs are in a metastable state. Most of those strongly bound by the correct codon–anticodon interaction and possessing L chirality descend to the peptidyltransferase center; however, most of those tRNAs weakly bound by incorrect codon–anticodon interactions or possessing D chirality dissociate. Although chemical and topological details of these states remain to be defined, in essence, proofreading depends on the balance of the rates of the binding, transpeptidation, and translocation cycle processes (Hopfield, 1974; Ninio, 1974, 1975; Blomberg, 1977).

EF-Tu·GTP appears to have several functions: the suppression of enzymatic and nonenzymatic hydrolysis of AA-tRNA; the hydrolysis of GTP; and the release of EF-Tu·GTP to free the aminoacyl stem of tRNA to generate the time delay and energy linkage essential to kinetic proofreading; considering the fact that the binding of AA-tRNA to the mRNA–ribosome complex can occur in the absence of EF-Tu (Pestka, 1969; Gavrilova et al., 1976; Spirin et al., 1976; Gavrilova & Spirin, 1971), one is tempted to speculate that EF-Tu was incorporated into the system, later in the evolution to accelerate

[3] The overall rates of dipeptide formation from the ternary complexes, EF-Tu·GTP·AA-tRNA, were calculated from the data in Figure 4A,D by subtracting the rate of peptide bond formation resulting from residual free (noncomplexed) AA-tRNA from the total rate of peptide bond formation. The rates resulting from free AA-tRNA were estimated as described in footnote 2. These rates were then normalized by dividing by the concentrations of ternary complexes calculated from their equilibrium constants.

the process enhancing the cell growth rate by increasing fidelity through kinetic proofreading of codon–anticodon interactions and chirality of the aminoacyl group and consequently producing a better chance for the species survival. It may also have eliminated D-amino acids from proteins at the same time.

Acknowledgments

The hexanucleotide used in the present experiment was prepared in Dr. O. C. Uhlenbeck's laboratory. We gratefully acknowledge the invaluable advice and technological help of Dr. O. C. Uhlenbeck and Dr. T. E. England.

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Uptake of Ornithine by Rat Liver Mitochondria†

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ABSTRACT: Uptake of [^{14}C]-L-ornithine by rat liver mitochondria has been measured by using the silicone sampling technique. The uptake of ornithine measured after 20–45 s of incubation exhibits stereospecificity, pH dependence, and a lack of dependence on respiratory energy. A slower subsequent increase in [^{14}C]-L-ornithine counts associated with the mitochondria, which is blocked by the transaminase inhibitor aminooxyacetate, is attributed to metabolism of the labeled ornithine. Each of the reagents *N*-ethylmaleimide, Tris (HCl) buffer, $Tl_2^+SO_4^{2-}$, $Mg^{2+}SO_4^{2-}$, and choline chloride inhibits ornithine accumulation. A lack of inhibition by mersalyl is interpreted as indicating that ornithine uptake does not require transmembrane P_i flux. Uptake of ornithine to levels in excess of the concentration in the medium can largely be accounted for by an osmotically insensitive fraction of the ornithine taken up, which is assumed to be adsorbed to solid structures of the mitochondria.

Mechanisms of uptake by mitochondria of various acidic and neutral amino acids have been extensively studied [e.g., see LaNoue & Schoolwerth (1979)]. Less is known about the transport of basic amino acids across mitochondrial membranes. Measurements of uptake of arginine by dog kidney mitochondria (Keller, 1968) and of lysine (King & Diwan, 1973; Diwan & Aram, 1974) and ornithine (Gamble & Lehninger, 1973; Bryla & Harris, 1976; McGivan et al., 1977)

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0006-2960/81/0420-7064$01.25/0