J. Biochem. **130**, 417–423 (2001)

Changing the Amino Acid Specificity of Yeast Tyrosyl-tRNA Synthetase by Genetic Engineering[1]

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Received May 28, 2001; accepted June 25, 2001

In an attempt to generate mutant aminoacyl-tRNA synthetases capable of charging non-canonical amino acids, a series of yeast tyrosyl-tRNA synthetase (TyrRS) mutants was constructed by site-specific mutagenesis of putative active site residues, which were deduced by analogy with those of *Bacillus stearothermophilus* TyrRS. Among these mutants, one with the replacement of tyrosine at position 43 by glycine, "Y43G," was found to be able to utilize several 3-substituted tyrosine analogues as substrates for aminoacylation. The catalytic efficiency (k_{cat}/K_m) of mutant Y43G for aminoacylation with L-tyrosine was about 400-fold decreased as compared to that of the wild-type TyrRS. On the other hand, the ability to utilize 3-iodo-L-tyrosine was newly generated in this mutant TyrRS, since the wild-type TyrRS could not accept 3-iodo-L-tyrosine at all under physiological conditions. This mutant TyrRS should serve as a new tool for site-specific incorporation of non-canonical amino acids, such as those in 3-substituted tyrosine analogues, into proteins in an appropriate translation system *in vivo* or *in vitro*.

Key words: amino acid specificity, aminoacylation, non-canonical amino acid, site-directed mutagenesis, tyrosyl-tRNA synthetase.

In recent years, a general strategy of site-specific incorporation of unnatural amino acids into proteins in a translation system *in vitro* has been developed (*1–3*). This method involves chemically acylated suppressor tRNAs, which incorporate non-canonical (*i.e.* not being specified in the genetic code) amino acids into the stop codons located at the predetermined position of the template mRNA. Although there have already been a large number of successful applications of this method for the incorporation of non-canonical amino acids into proteins (*4–7*), the usefulness of this system seems to be limited because it requires many steps of laborious biochemical and chemical treatments and therefore the final yields of the so-called "alloproteins" are typically not enough for sample-intensive methods, such as NMR spectroscopy.

One of the possibly effective solutions for circumventing such a problem would be to construct a mutant aminoacyl-tRNA synthetase (aaRS) capable of charging tRNA with non-canonical amino acid(s) and to utilize it in an *in vitro* translation system. However, it has not been an easy task to obtain such a mutant aaRS since the fidelity of this enzyme is of vital importance in the course of gene expression. Actually, the fidelity of this aminoacylation reaction is maintained at the level of substrate (both tRNA and amino acid) discrimination and in some cases, like isoleucyl- and valyl-tRNA synthetases, the editing (proofreading) mechanism is adapted to reject misacylated tRNAs (*8–11*). Although the principles by which aaRSs and their cognate tRNAs interact, so-called "tRNA identity," have recently become understood in detail (*12–14*), the mechanisms by which aaRSs recognize their cognate amino acids are less well characterized. The most notable exception to this would be the case of *Bacillus stearothermophilus* tyrosyl-tRNA synthetase (TyrRS), for which the discrimination mechanisms for the substrate L-tyrosine among other amino acids have been elucidated at the molecular level based on the crystal structures of TyrRS (*15–18*). A series of nondisruptive mutants carrying amino acid substitutions in the framework of active site residues of *B. stearothermophilus* TyrRS was constructed by Fersht and co-workers and was shown to have "relaxed" amino acid specificity (*18*, *19*).

We have been trying to utilize the yeast $tRNA^{Tyr}$/TyrRS pair as a possible candidate for the "carrier" of an extra amino acid in the *Escherichia coli* translation system *in vivo* (*20*) or *in vitro*, for the following reasons. (i) Yeast $tRNA^{Tyr}$ has a unique identity element, C1-G72 base pair, at the end of the acceptor stem (*21*, *22*), which is not found in *E. coli* tRNAs except for $tRNA^{Pro}$. (ii) The size of the variable arm of yeast $tRNA^{Tyr}$ is completely different from that of *E. coli* $tRNA^{Tyr}$, and thus, *E. coli* TyrRS will not aminoacylate yeast $tRNA^{Tyr}$. (iii) Yeast $tRNA^{Tyr}$ can be converted to amber or ochre suppressor tRNAs functional *in vitro* or *in vivo* (*23*, *24*). Since the crystal structure of yeast TyrRS has not yet been elucidated, we deduced its active site residues by comparison of the amino acid sequences of the *B. stearothermophilus* and yeast TyrRSs (*15*, *21*). Based on this information, we introduced several site-directed substi-

[1] This work was partly supported by Grants-in-Aid for the Encouragement of Young Scientists from the Japan Society for the Promotion of Science (to T.Y.).

[2] Satoshi Ohno was supported by Research Fellowships from the Japan Society for the Promotion of Science for Young Scientists.

Abbreviations: aaRS, aminoacyl-tRNA synthetase; L-DOPA, L-3,4-dihydroxyphenylalanine; IPTG, isopropyl-thio-β-D-galactoside; LysRS, lysyl-tRNA synthetase [EC 6.1.1.6]; TCA, trichloroacetic acid; TyrRS, tyrosyl-tRNA synthetase [EC 6.1.1.1]; OPA, *o*-phthalaldehyde; Y43G, replacement of tyrosine 43 to glycine.

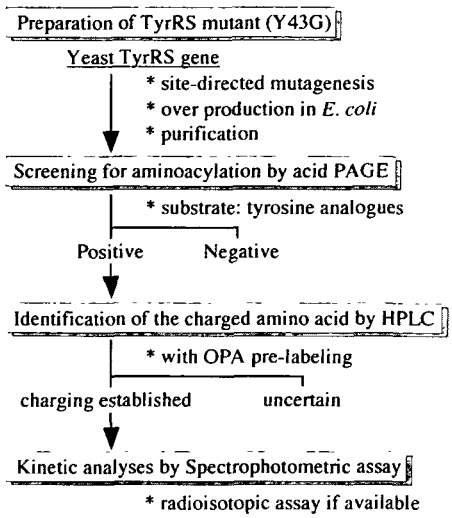

Fig. 1. **Schematic diagram of the overall experimental process.**

tutions into the presumed active sites of yeast TyrRS with the aim of obtaining a mutant yeast TyrRS which can aminoacylate $tRNA^{Tyr}$ (or its suppressor derivative) with non-canonical amino acid(s) instead of L-tyrosine.

We report in this paper that the substitution of tyrosine at position 43 to glycine (Y43G mutation) in yeast TyrRS lead to a drastic change in amino acid specificity. The overall experimental process is schematically shown in Fig. 1.

EXPERIMENTAL PROCEDURES

General—L- and D-tyrosine were obtained from Peptide Institute, and 3-azido-L-tyrosine was kindly provided by Drs. M. Suzuki and T. Hosoya (Gifu University). Other tyrosine analogues were from Sigma. Restriction enzymes were obtained from MBI Fermentas, and *Taq* DNA polymerase and Klenow fragment from Takara Shuzo. The reagents for spectrophotometric assaying, phosphoenolpyruvic acid, myokinase, pyruvate kinase, and lactate dehydrogenase were purchased from Roche Diagnostics, and NADH was from Oriental Yeast.

Preparation of Yeast $tRNA^{Tyr}$—Yeast $tRNA^{Tyr}$ was overproduced in *E. coli* BL21 (DE3) with plasmid pGEMEX-Tyr, and purified by ion-exchange column chromatographies. Plasmid pGEMEX-Tyr, containing the yeast $tRNA^{Tyr}$ gene, was constructed by the following method; two oligodeoxynucleotides containing 17 nucleotide-long complementary regions (underlined), 5′-GGG GTC TAG ACT CTC GGT AGC CAA GTT GGT TTA AGG CGC AAG ACT GTA AAT CTT GAG ATC GGG C-3′ and 5′-GGG GAA GCT TGG TCT CCC GGG GGC GAG TCG AAC GCC CGA TCT CAA GAT TT-3′, were annealed and converted to double-stranded DNA with Klenow fragment by incubation for 1 h at 37°C. The synthetic tRNA gene was digested with *Xba*I and *Hin*dIII, and then ligated to the *Xba*I and *Hin*dIII sites of pGEMEX-1 (Promega). The cell pellet was resuspended in 0.3 M sodium acetate (pH 4.75) and an equal volume of phenol equilibrated with H_2O was added. After shaking the mixture for 2 h, total RNAs were recovered by centrifugation. Yeast $tRNA^{Tyr}$ was isolated on Q-Sepharose HP (ϕ2 × 10 cm; Amersham Pharmacia Biotech.) with a linear gradient of NaCl (0.4 to 0.6 M) in 20 mM Tris-HCl (pH 7.6)–10 mM $MgCl_2$ (500 ml each), and subsequently in the second run on the same column with a linear gradient of NaCl (0.4 to 0.6 M) in 20 mM potassium acetate (pH 4.75)–10 mM $MgCl_2$ (500 ml each). This preparation of $tRNA^{Tyr}$ (about 70 A_{260} units per 1 liter culture) typically accepted 1,600 pmol L-tyrosine per 1 A_{260} unit under the standard assay conditions with the wild-type TyrRS.

Construction of Plasmids for Over-Expression of TyrRS and Mutant TyrRS—Plasmids pETTyrRS-His and pETY-43G-His were constructed by the following method. The coding region of yeast tyrosyl-tRNA synthetase was amplified by PCR from *Saccharomyces cerevisiae* (IFO1234) genomic DNA to create *Nde*I and *Xho*I sites at the 5′ and 3′ ends, and then inserted into pET21-a(+) (Novagen). The primers were 5′-GGG GGC ATG CAT ATG TCC TCT GCT GCC ACG GT-3′ (primer A) and 5′-GGG GCT GCA GCT CGA GCA ATT TGG TTT CCT-3′ (primer B) (*22*). The substitution of Y43 to G43 in TyrRS was made by the following method. First, the 5′ fragment of the yeast TyrRS gene was mutated and amplified using the polymerase chain reaction with primer A and 5′-GCG CGG TAC CCC AG*C* C*AA ATT TCA AAT GT-3′ (* = mismatched bases), and the 3′ fragment of the yeast TyrRS gene was mutated and amplified with 5′-ACA TTT GAA ATT TG*G* C*TG GGG TAC CGC GC-3′ (* = mismatched bases) and primer B. After amplification, these fragments were purified by agarose gel electrophoresis, and then annealed and converted to double-stranded DNA with Klenow fragment by incubation for 1 h at 37°C. Then the full-length mutated yeast tyrosyl-tRNA synthetase gene was amplified with primers A and B. Following amplification, the mutated yeast TyrRS gene was purified by agarose gel electrophoresis, digested with *Nde*I and *Xho*I, and then inserted into a pET21-a(+). The resulting plasmid, designated as pETY43G, contained an in-frame gene fusion between the yeast tyrosyl-tRNA synthetase gene with a Y43 to G43 replacement and a carboxyl-terminal hexa histidine-tag. The site of mutation was confirmed by dideoxy-DNA sequencing.

Purification of Wild-Type and Mutant TyrRSs—The histidine-tagged wild-type and Y43G mutant TyrRS proteins were purified as follows. BL21 (DE3) cells transformed with each vector were grown in LB-medium, supplemented with 50 mg/ml ampicillin with 0.5 mM IPTG when the cell density reached an A_{600} of 0.7. After 4 h, the cells were harvested by centrifugation and resuspended in buffer HM [20 mM Hepes-KOH (pH 7.3), 1 mM $MgCl_2$, 0.2 M NaCl, and 5% (v/v) glycerol]. After sonication, the cell debris was removed by centrifugation at 30,000 ×*g* for 45 min. The supernatant was applied to a Ni-NTA agarose column (Qiagen) and washed with buffer HM containing 10 mM imidazole, and then the histidine-tagged protein was eluted with buffer HM containing 250 mM imidazole. Moreover, the yeast wild-type and Y43G mutant TyrRSs were further purified by chromatography on Q-Sepharose HP (ϕ 1.5 × 9 cm) with a linear gradient of KCl (0 to 0.3 M) in 20 mM Hepes-KOH (pH 7.6) and 1 mM $MgCl_2$, and subsequently on SP-Sepharose HP (ϕ 1.5 × 9 cm; Amersham Pharmacia Biotech.) with a linear gradient of KCl (0 to 0.5 M) in 20 mM Hepes-KOH (pH 7.0) and 1 mM $MgCl_2$. Fractions containing TyrRS or a mutant were pooled and concentrated with the use of a Centri-Prep 10 (Millipore), and then dialyzed against storage buffer [Tris-HCl (pH 7.6), 1 mM

$MgCl_2$, 40 mM KCl, and 50% glycerol) and stored at −20°C. The concentrations of the purified enzymes were determined by the method of Ehresmann *et al.* (*25*).

Screening of Aminoacylation with Tyrosine Analogues—The aminoacylation mixture (total volume, 10 μl) contained 100 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 40 mM KCl, 4 mM ATP, 200 μM tyrosine analogues, 0.2 μg TyrRS, and 0.02 A_{260} units tRNA. The reaction was carried out at 30°C for 10 min and was stopped by lowering the pH to 4.75 with 6% acetic acid, and then aliquots were subjected to electrophoresis on a 10% polyacrylamide slab gel under acidic conditions (pH 4.75). The RNA bands were visualized by staining with methylene blue.

Analysis of Amino Acids and Identification of Charged Amino Acids—Aminoacyl-tRNAs were isolated by anion exchange column chromatography with a stepwise NaCl gradient elution under acidic conditions (pH 4.75), and precipitated with ethanol. Analyses of fluorescence-labeled amino acids were carried out according to the method of Jones *et al.* (*26*), using a Hitachi HPLC instrument with a TSKgel Super-ODS (4.6 mmID × 10 cm; Tosoh) column with a sample load of 30 μl and monitored at 455 nm (emission at 455 nm, excitation at 340 nm) with an Intelligent Fluorescence Detector (JASCO). Separation was performed with a linear gradient from Buffer A [THF:methanol:50 mM sodium acetate (pH 5.9) = 1:19:80 (v/v)] to Buffer B (methanol: 50 mM sodium acetate (pH 5.9) = 80:20 (v/v)]. For the analysis of D-tyrosine, *N*-acetyl-L-cysteine was used instead of 2-mercaptoethanol.

Determination of Kinetic Parameters by Spectrophotometric Assaying—Spectrophotometric assaying was carried out essentially according to the method of Wu and Hill (*27*), except that the reaction conditions were optimized for the yeast TyrRS. The absorbance at 340 nm (A_{340}) was monitored against a reference cuvette containing all the components except TyrRS with a UV/Vis spectrophotometer (Pharmacia) equipped with a thermostatically controlled cell holder set at 30°C. The initial rates were determined at various tyrosine or analogue concentrations from the time course. Kinetic parameters were obtained from Lineweaver-Burk plots.

RESULTS AND DISCUSSION

Preparation of Wild-Type and Mutant TyrRSs—As a possible candidate for generating a mutant aminoacyl-tRNA synthetase which can catalyze esterification of the 3′ terminal hydroxyl group of tRNA with non-canonical amino acid(s), we chose the yeast $tRNA^{Tyr}$/TyrRS pair. The rationale for this choice was that yeast $tRNA^{Tyr}$ cannot be aminoacylated by *E. coli* TyrRS, nor *E. coli* $tRNA^{Tyr}$ by yeast TyrRS (*28–31*), and thus the yeast $tRNA^{Tyr}$/TyrRS pair was expected to act as an extra tRNA/aaRS set for non-canonical amino acids in an *E. coli* cell-free protein synthesizing system. Since the crystal structure of yeast TyrRS has not yet been solved, we inferred its active site residues by comparison of the amino acid sequences of the *Bacillus stearothermophilus* and yeast TyrRSs (*15–19*, *21*). Based on this comparison, we constructed several point-mutants by introducing site-directed substitutions into the putative active site residues in yeast TyrRS, which are comparable to those in *B. stearothermophilus* TyrRS. Although most of these mutations resulted in a failure to produce proteins of desired molecular weights or a total loss of catalytic activity (data not shown), one of the mutants, named Y43G, in which the tyrosine residue at position 43 (corresponding to position 34 in *B. stearothermophilus* TyrRS; see Fig. 2) was replaced by glycine, was found to be a possible candidate.

In order to obtain a reasonable expression yield of the wild-type or mutant TyrRS, we employed an expression system based on the transcription system utilizing T7 RNA polymerase (*32*). To facilitate purification and monitoring of the expression of TyrRS, the enzymes were designed so as to be tagged with six histidine residues at the carboxyl terminus. Chromatography on a Ni-NTA column and the use of Ni-NTA alkaline phosphatase for the detection of histidine-tagged TyrRSs proved to be expectedly useful for rapid and efficient purification of the desired protein products (data not shown).

Aminoacylation Properties of Histidine-Tagged TyrRS—Since the prepared enzymes each possessed an extra histidine-tag at the carboxyl terminus, the effect of an extra histidine-tag on the catalytic behavior of each enzyme had to be examined. Kinetic parameters of tyrosylation by a preparation of wild-type TyrRS (plus a histidine-tag) expressed in *E. coli* cells were determined by two separate methods (Table I). One was a conventional radioisotopic method involving [^{14}C]-L-tyrosine and trichloroacetic acid (TCA) precipitation. The other was a spectrophotometric method essentially based on the method of Wu and Hill (*27*). As shown in Table I, the K_m and k_{cat} values for tyrosylation obtained with both methods were essentially in good agreement with those reported previously for native yeast TyrRS (without histidine-tag). Thus, the extra six histidine resi-

Fig. 2. **Comparison of partial amino acid sequences of *Bacillus stearothermophilus* (Bs) and *Saccharomyces cerevisiae* cytoplasmic (Sc) tyrosyl-tRNA synthetases (*21*).** Only the local sequences that are conserved and supposed to participate in the discrimination of L-tyrosine are shown. The asterisk (*) indicates the mutated position in this work (Y34 in Bs and Y43 in Sc).

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                                  *
Bs 18 DE----DGLRKLLN-EER-VTLYCGFDPTADSLHIGHLATILTMRRFQQA 61
Sc 21 QEVLNPQIIKDVLEVQKRHLKLYWGTAPTG-RPHCGYFVPMTKLADFLKA 69
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TABLE I. **Comparison of kinetic parameters between native and histidine-tagged yeast TyrRSs.**

TyrRS	Method[a]	K_m (mM)	k_{cat} (min^{-1})	Reference
Native	RI	0.012	308.4	Freist and Sternbach (*34*)
	RI	0.005		Kijima *et al.* (*35*)
Recombinant (plus His-tag)	RI	0.012 ± 0.001	262 ± 12	This study
	SP	0.014 ± 0.001	276 ± 48	This study

[a] RI: radioisotopic, SP: spectrophotometric.

dues at the carboxyl terminus were shown to have little influence, if any, on the aminoacylation process, at least when L-tyrosine was used as the substrate. Nishimura and co-workers (*33*) have generated an *E. coli* TyrRS mutant capable of charging azatyrosine to tRNA. They also reported that *E. coli* wild-type TyrRS exhibited the same specific activity in the histidine-tagged and unmodified forms.

Assaying of Aminoacylation by Acid PAGE—As a screening assay methed for the ability of tyrosine analogues to act as substrates for the wild type and Y43G mutant TyrRSs, electrophoretic separation on a acid polyacrylamide gel was exploited for discrimination between aminoacylated and non-acylated tRNAs after the aminoacylation reaction. Figure 3 shows typical results of such analysis with L-tyrosine and some tyrosine analogues as substrates. Some of the tRNA bands are apparently delayed, suggesting that Y43G mutant TyrRS can possibly utilize these tyrosine analogues for the aminoacylation of tRNA. The results of similar assays with more than 40 species of tyrosine analogues are summarized in Table II, column A. In addition to L-tyrosine, analogue numbers 1 (D-tyrosine)–10 (3-azido-L-tyrosine) gave positive signals in this assay. When wild-type TyrRS was used for the same assay, marginally positive signals were observed for analogue numbers 23, 27–32, 34, and so on (data not shown; T. Yokogawa *et al.*, unpublished observation). However, these signals might have been false ones because slight contamination by L-tyrosine of the amino acids used for the aminoacylation assay was detected (Table II, column B). The reason why such marginally positive signals were not observed in the assay with Y43G mutant TyrRS (Table II, column A) would be the finding that the catalytic efficiency (k_{cat}/K_m) of the mutant TyrRS for aminoacylation with L-tyrosine as a substrate was decreased about 400-fold compared to that of wild-type TyrRS, as discussed below.

TABLE II. **Summary of aminoacylation reactions with tyrosine analogues by Y43G mutant TyrRS.**

		Method of analysis		
		A	B	C
1.	D-tyrosine	+	−	+
2.	*m*-fluoro-D,L-tyrosine	+	−	+
3.	3-chloro-L-tyrosine	+	−	+
4.	3-iodo-L-tyrosine	+	−	+
5.	3-amino-L-tyrosine	+	−	*
6.	L-3,4-dihydroxyphenylalanine (L-DOPA)	+	−	+
7.	D-3,4-dihydroxyphenylalanine (D-DOPA)	+	−	
8.	3-methoxy-L-tyosine	+	±	±
9.	3-nitro-L-tyosine	+	−	*
10.	3-azido-L-tyosine	+	−	*
11.	6-hydroxy DOPA	−		
12.	D,L-*o*-tyrosine	−		
13.	D,L-*m*-tyrosine	−		
14.	3,5-diiodo-L-tyrosine	−		
15.	3,5-dibromo-L-tyrosine	−		
16.	3,5-dinitro-L-tyrosine	−		
17.	L-phenylalanine	−		
18.	*p*-fluoro-L-phenylalanine	−		
19.	*p*-fluoro-D-phenylalanine	−		
20.	*p*-iodo-phenylalanine	−		
21.	*p*-amino-L-phenylalanine	−	−	
22.	*p*-amino-D-phenylalanine	−		
23.	*O*-methyl-L-tyrosine	−	+	
24.	*p*-nitro-L-phenylalanine	−		
25.	3,5-diiodo-L-thyronine	−		
26.	3,3,5-triiodo-L-thyronine	−		
27.	*O*-phospho-L-tyrosine	−	+	
28.	*O*-mono-2,4-DNP-L-tyrosine	−	+	
29.	*O*-dansyl-L-tyrosine	−	+	
30.	*N*-*o*-nitrophenylsulfenyl-L-tyrosine	−	+	
31.	*N*-acetyl-L-tyrosine	−	+	
32.	*N*-formyl-L-tyrosine	−	+	
33.	L-tyroxine	−		
34.	α-methyl-L-*p*-tyrosine	−	−	
35.	L-(+)-*p*-hydroxyphenylglycine	−		
36.	tyramine	−		
37.	L-tyrosinol	−		
38.	3-(*p*-hydroxyphenyl)propionic acid	−		
39.	D,L-*p*-hydroxyphenyllactic acid	−		
40.	L-tyrosinamide	−	−	
41.	L-β-phenyllactic acid	−		
42.	L-3-(1-naphthyl)alanine	−		
43.	L-3-(2-naphthyl)alanine	−		
	L-tyrosine	+	+	+

A: Aminoacylation assaying by acid PAGE. +, positive signal of aminoacylation suggested by this analysis. −, no indication of aminoacylation. B: Analysis of L-tyrosine contamination of the amino acid samples by HPLC (with OPA pre-labeling). +, contamination suggested by this analysis; ±, OPA-labeled product not separable from OPA-L-tyrosine; −, no contamination detected; blank, not done. C: Analysis of the "charged" amino acid by HPLC (with OPA pre-labeling). +, aminoacylation established by this analysis; *, analysis impossible because of difficulty in OPA pre-labeling; ±, OPA-labeled product not separable from OPA-L-tyrosine; blank, not done.

Identification of the "Charged" Amino Acids—Although acid PAGE is rapid and convenient for screening amino acid candidates utilized for aminoacylation by the mutant (and wild type) enzymes, one must be careful in interpret-

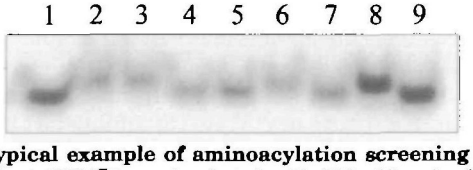

Fig. 3. **Typical example of aminoacylation screening by acid PAGE.** Yeast $tRNA^{Tyr}$ was incubated with 200 μM each of tyrosine analogues and Y43G mutant TyrRS (lanes 2–7), or with 200 μM L-tyrosine and wild type TyrRS (lane 8). Aliquots of each reaction mixture were subjected to acid PAGE and the RNA bands were visualized by staining with methylene blue. Lanes 1 and 9, no TyrRS; lane 2, L-DOPA; lane 3, 3-iodo-L-tyrosine; lane 4, 3,5-dinitro-L-tyrosine; lane 5, 5-diiodo-L-tyrosine; lane 6, 3-amino-L-tyrosine; lane 7, 3,5-dibromo-L-tyrosine; lane 8, L-tyrosine (positive control).

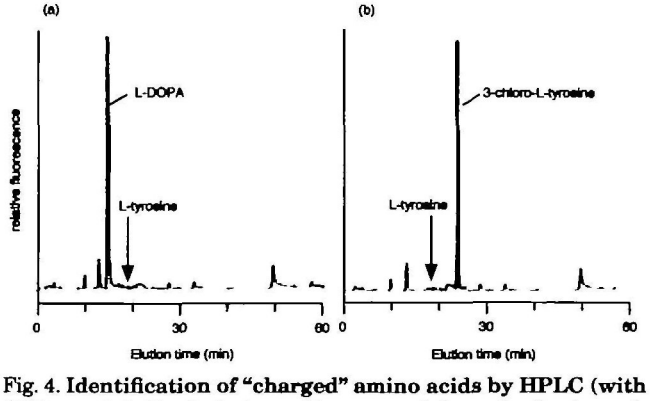

Fig. 4. **Identification of "charged" amino acids by HPLC (with OPA pre-labeling).** Amino acids prepared from the "aminoacyl-tRNA" derived from (a) L-DOPA or (b) 3-chloro-L-tyrosine were labeled with OPA and then separated by HPLC (Super ODS column). The arrows indicate the elution position of L-tyrosine.

ing the results. Since aminoacylation is judged with the acid PAGE system only as to the relative positions of tRNA bands on the gel, it is rather dangerous to believe that the delayed tRNA is actually charged with the amino acids of interest. As considerably high concentrations (200 μM) of tyrosine analogues were used in the assay, even a small level (*e.g.* about 1%) of contaminating L-tyrosine would give an apparently positive signal. There is also the possibility that some of the tyrosine analogues might be transformed or degraded into L-tyrosine during the aminoacylation reaction. Therefore, the amino acids shown to be "positive" by the acid PAGE assay were subjected to further analysis by HPLC after detaching the aminoacyl-tRNA and OPA-labeling. Typical examples of such analyses are shown in Fig. 4. Authentic OPA-labeled L-tyrosine was eluted at 18 min (Fig. 4, arrow) under the elution conditions used. The amino acid sample prepared from the "aminoacyl-tRNA" derived from L-DOPA was eluted at 15.2 min (Fig. 4a) corresponding to the authentic L-DOPA, and thus it was identified as L-DOPA. Similarly, 3-chloro-L-tyrosine (Fig. 4b), D-tyrosine, 3-fluoro-D,L-tyrosine, and 3-iodo-L-tyrosine (and L-tyrosine, of course) were identified by this analysis (Table II, column C). On the other hand, 3-amino-L-tyrosine, 3-nitro-L-tyrosine, and 3-azido-L-tyrosine did not give any positive signal in this analysis (Table II, column C). However, since authentic amino acids of this group were somehow difficult to label with OPA, the absence of a positive signal in this analysis does not necessarily mean that these tyrosine analogues cannot be substrates for aminoacylation. The results of analyses in Table II, column B, indicate no evidence of contamination by L-tyrosine of these amino acids. A new method is needed to see if these latter amino acids were actually esterified to the tRNA or not. As for 3-methoxy-L-tyrosine, an unambiguous result was not obtained since the OPA-labeled product was eluted at a position very close to that of OPA-L-tyrosine (Table II, columns B and C).

Spectrophotometric Analysis of Aminoacylation Kinetics—In order to obtain kinetic parameters for aminoacylation using the tyrosine analogues as substrates, there was no alternative but to use a non-radioisotopic assay method, even though it is sample-intensive, since most of the analogues were not available in radioisotopically labeled forms. We chose the spectrophotometric assay method of Wu and Hill (27), and optimized the reaction conditions for yeast TyrRS. Figure 5 shows typical examples of such measurements. Absorbance at 340 nm began to decrease as soon as Y43G mutant TyrRS was added to the reaction mixture containing $tRNA^{Tyr}$ and 3-fluoro-D,L-tyrosine (100 μM or 1 mM), whereas the reaction mixture lacking the amino acid did not show such a decrease (Fig. 5a). A similar time course when 1.4 mM 3-iodo-L-tyrosine was used as the substrate in the presence of wild-type or Y43G mutant TyrRS is shown in Fig. 5b. The latter data clearly indicate that 3-iodo-L-tyrosine can be a substrate for the Y43G mutant but not for wild-type TyrRS. The apparent kinetic constants (K_m, k_{cat}) were derived from Lineweaver-Burk plots. The data summarized in Table III represent the averages of at least two independent experiments. The kinetic parameters (K_m, k_{cat}, and k_{cat}/K_m) for L-tyrosine and wild type TyrRS obtained by this method are in good agreement with those obtained by the conventional radioisotopic assay method (Table I).

Change of Aminoacylation Properties—As can be seen from Tables II and III, Y43G mutant TyrRS can use some of the 3-substituted tyrosine analogues as substrates for aminoacylation of $tRNA^{Tyr}$. Although 3-bromo-L-tyrosine was not tested due to its unavailability, it would certainly

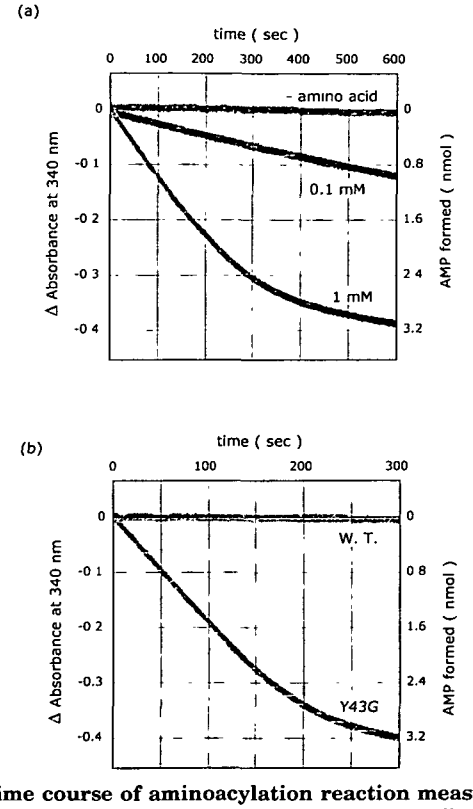

Fig. 5. **Time course of aminoacylation reaction measured by spectrophotometry.** The assay conditions are essentially based on the method of Wu and Hill (27). Progress of the reaction (at 30°C) was monitored as the decrease in A_{340} (NADH), which is proportional to the formation of AMP. (a) With 3-fluoro-D,L-tyrosine (varying concentrations) and Y43G mutant TyrRS. (b) With 3-iodo-L-tyrosine (1.4 mM) and wild type (W.T.) or Y43G mutant TyrRS.

TABLE III. **Kinetic parameters for aminoacylation of yeast tRNATyr by the yeast wild-type TyrRS and Y43G mutant.**

Amino acid	K_m (mM)		k_{cat} (s^{-1})		k_{cat}/K_m (× mM^{-1} s^{-1})	
	W.T.	Y43G	W.T.	Y43G	W.T.	Y43G
L-tyrosine	0.014 ± 0.001	1.19 ± 0.14	4.6 ± 0.8	0.95 ± 0.02	321	0.804
D-tyrosine	0.459 ± 0.014	14.0 ± 0.04	1.2 ± 0.02	0.37 ± 0.03	2.62	0.027
3-fluoro-D,L-tyrosine	0.625 ± 0.005	1.3 ± 0.09	4.96 ± 0.3	0.62 ± 0.09	7.97	0.475
3-chloro-L-tyrosine	2.04 ± 0.25	2.43 ± 0.03	0.13	1.46 ± 0.12	0.065	0.602
3-iodo-L-tyrosine	N.D.	1.15 ± 0.15	N.D.	0.48 ± 0.02	N.D.	0.425
L-DOPA	1.84 ± 0.3	0.56 ± 0.01	1.65 ± 0.3	0.67 ± 0.06	0.895	1.020

N.D.: Reaction not detected.

be a substrate since all other 3-halogenated tyrosine analogues are considerably good substrates for this mutant TyrRS. It should be noted that L-DOPA (L-3,4-dihydroxyphenylalanine) is also one of the 3-substituted tyrosine analogues since it is equivalent to 3-hydroxy-L-tyrosine. The wild-type TyrRS accepted 3-fluoro-D,L-tyrosine as a substrate even more than Y43G mutant TyrRS, but the catalytic efficiency (k_{cat}/K_m) of the wild-type TyrRS for 3-chloro-L-tyrosine was about one-tenth of that of the Y43G mutant TyrRS, and as for 3-iodo-L-tyrosine, the wild-type TyrRS could not accept it at all, while the Y43G mutant could (Table III and Fig. 6). All of these data are well explained by assuming that a free space accommodating the 3-substituted group was generated by the tyrosine 43 to glycine replacement around the tyrosine binding pocket of TyrRS. Position 43 of yeast TyrRS corresponds to position 34 of *B. stearothermophilus* TyrRS (Fig. 2), and the side chain of tyrosine 34 constitutes one side of the substrate (L-tyrosine) binding pocket in *B. stearothermophilus* TyrRS (*15*). The catalytic efficiency (k_{cat}/K_m) of Y43G mutant TyrRS for aminoacylation with the original substrate, L-tyrosine, was decreased about 400-fold compared to that of the wild-type TyrRS (0.804 *vs.* 321), and the Y43G mutant TyrRS has a greater tolerance for 3-substituted tyrosine analogues compared to that of the wild-type TyrRS.

Use of the Y43G Mutant for Specialized Protein Synthesis—We have been examining the possibility of utilizing the yeast $tRNA^{Tyr}$/TyrRS pair as a "carrier" of an extra amino acid in the *E. coli* translation system *in vivo* (*20*) or *in vitro*. When used together with yeast suppressor $tRNA^{Tyr}$, this mutant, Y43G, should serve as an effective tool for site-specific incorporation of 3-substituted tyrosine analogues into proteins in an appropriate *E. coli* translation system. However, care must be taken in the use of yeast suppressor $tRNA^{Tyr}$, because it is possibly misaminoacylated by other *E. coli* aaRSs, even though it is not aminoacylated by *E. coli* TyrRS. We found that the suppressor $tRNA^{Tyr}$ could be aminoacylated by *E. coli* LysRS depending on the balance of the levels of enzymes and tRNAs. This might have been the cause of the discrepancy between the recent report of Wang *et al.* (*36*) and ours (*20*). The expression of the suppressor $tRNA^{Tyr}$ was kept purposely at a low level by using low-copy-number vector pMW118 (Nippon Gene) in our experiment (*20*), but controlling the expression level is not an easy task. We have now succeeded in generating a mutant suppressor $tRNA^{Tyr}$ which is practically not aminoacylated by *E. coli* LysRS by substituting several nucleotide residues in the tRNA molecule (J. Fukunaga *et al.*, unpublished results).

Recently, RajBhandary and co-workers (*37*) described two aaRS/suppressor tRNA pairs for possible use in site-specific incorporation of amino acid analogues into proteins, one for use in yeast and the other for use in *E. coli*. They found that wild-type yeast TyrRS has a tendency to misaminoacylate *E. coli* $tRNA^{Pro}$, and also described the isolation of three TyrRS mutants which show increased discrimination *in vitro* for the suppressor tRNA over the *E. coli* $tRNA^{Pro}$. Curiously, our preparation of wild-type yeast TyrRS exhibited no such ability to aminoacylate *E. coli* $tRNA^{Pro}$ when assayed *in vitro* with purified *E. coli* $tRNA^{Pro}$ (data not shown). However, the DNA sequence of our wild-type yeast TyrRS gene is different from their sequence (*21*) in only one nucleotide, which would bring about an asparagine (theirs)-to-aspartic acid (ours) change at position 343 of the enzyme. It is very interesting that one of their mutant TyrRSs, TyrRS*9, has an amino acid change at position 344 from asparagine to histidine (*37*). Although the reason why the sequences of the two "wild-type" TyrRS are different is not clear, it is probable that the asparagine at position 343 and/or 344 is somehow involved in the misaminoacylation of *E. coli* $tRNA^{Pro}$.

One of the promising applications of this mutant TyrRS will be for structural determination of proteins by X-ray crystallography. In this field, a powerful method (Multiple Wavelength Anomalous Dispersion: MAD) involving a selenomethionine-substituted protein is currently prevailing (*38*). We think 3-bromo-L-tyrosine could be an alternative to selenomethionine since bromine has a very convenient X-ray absorption edge at 0.92 Å, which is similar to that of selenium (0.98 Å), and bromouracil has been used for MAD phasing of nucleic acids following the substitution of almost isostructural thymine. Although we could not include 3-bromo-L-tyrosine as a screening candidate for aminoacylation in this study, we believe it can certainly be a substrate for Y43G mutant TyrRS. Once it is esterified to the 3′ end of suppressor $tRNA^{Tyr}$, we can incorporate a bromine-containing residue(s) into any positions of the target protein at will by designing the location of amber codon(s) within the template mRNA for *in vitro* protein synthesis. Also promising would be the use of 3-azido-L-tyrosine for cross-linking studies. As already mentioned above, we could not firmly establish 3-azido-L-tyrosine as a substrate for Y43G mutant TyrRS because it could not be labeled efficiently with OPA. However, it gives a positive signal on acid PAGE analysis

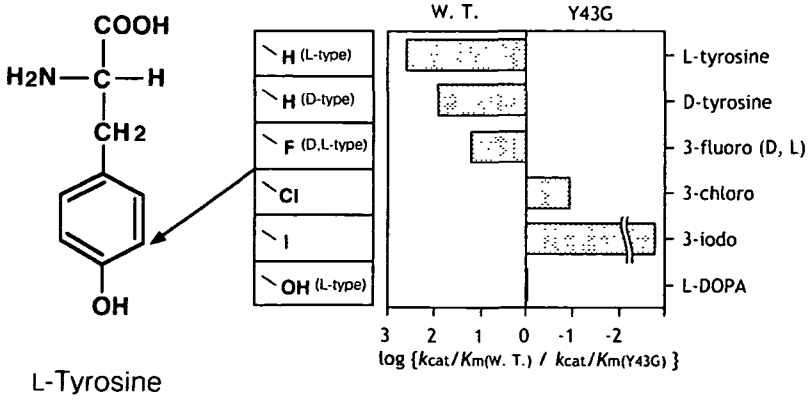

Fig. 6. **Change of amino acid specificity by genetic engineering of yeast tyrosyl-tRNA synthetase.** Left, tyrosine analogues that are able to be substrates for Y43G mutant TyrRS; Right, change of aminoacylation efficiency of individual tyrosine analogues, as indicated by the value log $\{k_{cat}/K_m(W.T.)/k_{cat}/K_m(Y43G)\}$.

and no evidence of contamination by L-tyrosine was indicated in this preparation of 3-azido-L-tyrosine (kind gift from Drs. M. Suzuki and T. Hosoya, Gifu University). A preliminary cross-linking study involving 3-azido-L-tyrosyl tRNA is underway in our laboratory.

The authors wish to thank Dr. Haruichi Asahara of this laboratory and Dr. Nobutada Tanaka of Showa University for the valuable suggestions.

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