A General Method for Site-Specific Incorporation of Unnatural Amino Acids into Proteins

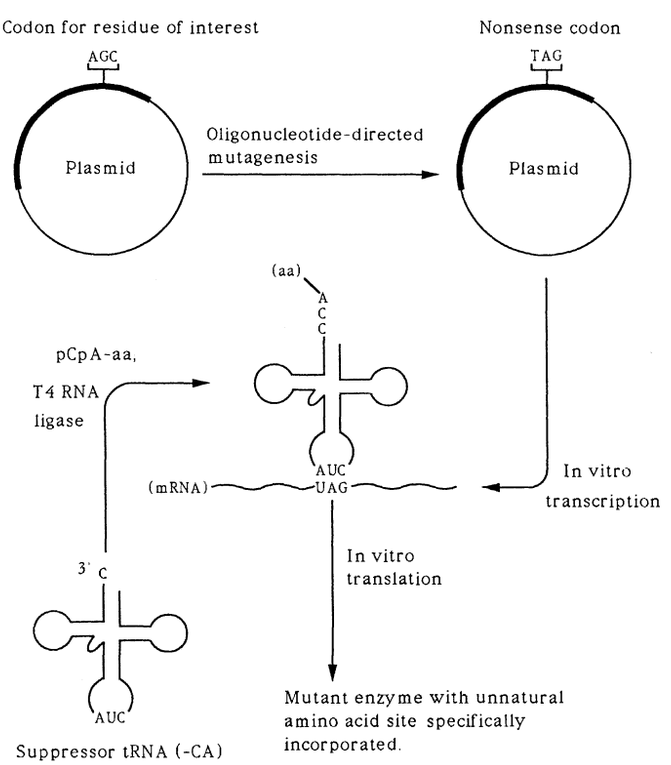
CHRISTOPHER J. NOREN, SPENCER J. ANTHONY-CAHILL, MICHAEL C. GRIFFITH, PETER G. SCHULTZ*

A new method has been developed that makes it possible to site-specifically incorporate unnatural amino acids into proteins. Synthetic amino acids were incorporated into the enzyme β-lactamase by the use of a chemically acylated suppressor transfer RNA that inserted the amino acid in response to a stop codon substituted for the codon encoding residue of interest. Peptide mapping localized the inserted amino acid to a single peptide, and enough enzyme could be generated for purification to homogeneity. The catalytic properties of several mutants at the conserved Phe^{66} were characterized. The ability to selectively replace amino acids in a protein with a wide variety of structural and electronic variants should provide a more detailed understanding of protein structure and function.

RECENT ADVANCES IN MOLECULAR BIOLOGY HAVE MADE IT possible to substitute any amino acid in a protein with one of the other 19 natural amino acids. Characterization of the resulting mutant proteins has increased our insight into the nature of molecular recognition and catalysis in biological systems (*1*). However, the range of useful substitutions is limited. For example, few substitutions that are sterically (Asp to Asn) or functionally (Asp to Glu) equivalent can be made with the 20 natural amino acids. Ideally, one would like to tailor the structure of an amino acid to address a specific structure-function relation. Such substitutions might include alterations in the acidity or nucleophilicity of enzyme active site residues, introduction of electron acceptors or metal chelators into proteins, alteration of hydrogen bond donor or acceptor functionality in DNA binding proteins, or introduction of amino acids with altered or restricted torsion angles. The ability to selectively replace amino acids in proteins with a wide variety of structural variants should allow us to understand protein structure and function in more detail.

A number of methods currently exist that allow unnatural amino acids to be incorporated into proteins or peptides. Peptide synthesis (*2*) and semisynthetic methods (*3*) have been used to substitute novel amino acids into small proteins (<10 kD) and peptides. Chemical modification has also been used to modify unusually reactive amino acid side chains that are solvent accessible (*4*). Modified amino acids have been uniformly incorporated into peptides and proteins with functional analogues of aminoacyl transfer RNA's (tRNA's) (*5, 6*). In addition, several unnatural amino acids have been incorporated into dipeptides through the use of chemically misacylated tRNA's (*7*). These methods all suffer either from nonselective introduction of the novel amino acid or from size restrictions on the protein of interest.

We report a general biosynthetic method that makes it possible to site-specifically incorporate unnatural amino acids with novel steric and electronic properties into proteins (scheme 1 below). Our approach involves replacement of the codon encoding the amino acid of interest with the "blank" nonsense codon, TAG, by oligonucleotide-directed mutagenesis. A suppressor tRNA directed against this codon is then chemically aminoacylated in vitro with the desired unnatural amino acid. Addition of the aminoacylated tRNA to an in



The authors are at the Department of Chemistry, University of California, Berkeley, CA 94720, and the Center for Advanced Materials, Lawrence Berkeley Laboratory, Berkeley, CA 94720.

*To whom correspondence should be addressed.

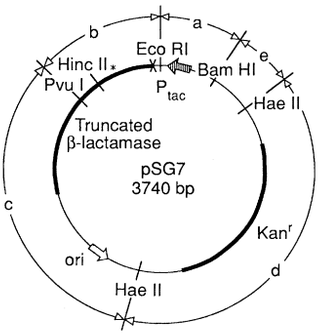


Fig. 1. Construction of the plasmid pSG7, the vector for in vitro expression of β-lactamase. Segment a is the 259-bp Bam HI–Eco RI fragment of pKK223-3 (*52*), containing the *tac* promoter. Segment b is the 376-bp Ssp I (linked to Eco RI)–Pvu I fragment of pTG2*del*1 (*23*) containing the first part of the gene for RTEM β-lactamase (*14*) with a 63-bp deletion corresponding to 21 amino acids in the leader sequence. Segment c is the 1386-bp Pvu I–Hae II fragment of pT7-3 (*26*), containing the remainder of the β-lactamase gene and the ColE1 origin of replication. Segment d is the 1430-bp Hae II–Hae II fragment of pGP1-2 (*26*), containing the kanamycin resistance gene from Tn*903* (*29*). This gene is oriented so as not to be under the transcriptional control of the *tac* promoter. Segment e is the 289-bp Hae II–Pvu II (ligated to the blunt-ended Bam HI site of segment a to regenerate only the Bam HI site) fragment from pT7-3. Mutants at Phe^{66} (* on figure) were generated with the method of Eckstein (*53*). The 204-bp Eco RI–Hinc II fragment, containing the codon for Phe^{66}, was cloned into M13mp18. Three synthetic oligodeoxynucleotides, 5′-ATCATTGGATAACGTTCTT-3′, 5′-ATCATTGGAGCACGTTCTT-3′, and 5′-ATCATTGGCTAACGTTCTT-3′ (underlined bases denote mismatches to the wild-type sequence) were used to generate the F66Y, F66A, and F66*am* mutants, respectively. Mutagenesis efficiencies were 100 percent for Tyr^{66}, 83 percent for Ala^{66} and 60 percent for TAG^{66}.

Table 1. Characterization of native and mutant β-lactamases. Wild-type Phe^{66}-suppressed β-lactamases were purified to homogeneity from 1-ml in vitro reactions primed with pSG7 and pF66*am*/Phe-tRNA$^{Phe}_{CUA}$ respectively. Initial rates of nitrocefin hydrolysis were determined, at 24°C in 50 m*M* sodium phosphate, *p*H 7, 0.5 percent DMSO, at substrate concentrations ranging from 25 to 250 μ*M*. K_m and maximum velocity V_{max} values were obtained from Eadie-Hofstee plots, and Bradford assay quantitations of the enzymes were used to determine k_{cat} values. Kinetic parameters for the mutant enzymes were determined as follows: In vitro reactions (60 μl) containing 15 μCi of [^{35}S]Met (Amersham) were primed with pSG7, pF66Y, pF66*am*/Phe-tRNA$^{Phe}_{CUA}$, pF66*am*/*p*-FPhe-tRNA$^{Phe}_{CUA}$, pF66*am*/*p*-NO_2Phe-tRNA$^{Phe}_{CUA}$, pF66*am*/HPhe-tRNA$^{Phe}_{CUA}$, pF66*am*/PLA-tRNA$^{Phe}_{CUA}$, pF66*am*/ABPA-tRNA$^{Phe}_{CUA}$, or pF66*am*/D-Phe-tRNA$^{Phe}_{CUA}$ and incubated at 37°C for 30 minutes. K_m and V_{max} values were determined as described above; the crude enzyme was used immediately following incubation. Quantitation of the enzymes was achieved by first precipitating the crude reaction with trichloroacetic acid and washing at 90°C to remove unincorporated label (*22*). The measured incorporated radioactivity for the Phe^{66} enzyme was then used, together with the k_{cat} value of 880 s^{-1} determined from Bradford assay quantitation, to calculate the amount of incorporated radioactivity per micromole of enzyme (typically 5 mCi/μmol). This value was then used to quantitate the mutant enzymes on the basis of the incorporated radioactivity measured for each. Values shown are the averages of three determinations.

Amino acid	Suppressor	Enzyme synthesized* (μg/ml)	K_m (μ*M*)	k_{cat} (s^{-1})
Phe	–	26.0 ± 3.8	55 ± 5	880 ± 10†
Phe	Phe-tRNA$_{CUA}$	2.9 ± 0.9	59 ± 6	870†
Tyr	–	16.9 ± 2.3	49 ± 3	420 ± 40*
p-FPhe	*p*-FPhe-tRNA$_{CUA}$	2.1 ± 0.9	59 ± 2	1120 ± 290*
p-NO_2Phe	*p*-NO_2Phe-tRNA$_{CUA}$	3.0 ± 1.0	57 ± 4	370 ± 70*
HPhe	HPhe-tRNA$_{CUA}$	1.0 ± 0.4	72 ± 14	150 ± 60*
PLA	PLA-tRNA$_{CUA}$	0		
ABPA	ABPA-tRNA$_{CUA}$	0		
D-Phe	D-Phe-tRNA$_{CUA}$	0		

*Based on incorporated radioactivity (see legend). †Based on Bradford assay quantitation of purified enzyme.

vitro protein synthesizing system programmed with the mutagenized DNA directs the insertion of the prescribed amino acid into the protein at the target site.

The development of this strategy was based on the following experimental observations. The nonsense codon TAG can be suppressed relatively efficiently in vitro and in vivo by naturally occurring or semisynthetic amber suppressor tRNA's (*8, 9*). Anticodon-codon recognition has been shown to be independent of the amino acid at the acceptor stem of the tRNA (adapter hypothesis), indicating that noncognate amino acids should be incorporated into protein (*10*). Elongation factor and peptidyltransferase show relatively broad substrate specificity, suggesting that a wide variety of amino acid side chains can be accommodated by the ribosome. Finally, Hecht and co-workers have developed a chemical strategy for aminoacylating tRNA's with N-blocked amino acids (*11*). Brunner has extended this chemistry so that the chemically aminoacylated tRNA's can also function in the A site of the ribosome, thereby making it possible to avoid the specificity of the naturally occurring aminoacyl-tRNA synthetases (*6*).

Mutagenesis and in vitro protein synthesis. The mutagenesis methodology described above has been developed with the well-characterized hydrolytic enzyme, RTEM β-lactamase (*12, 13*). This bacterial (*Escherichia coli*) enzyme is a single-chain 29-kD protein that contains one disulfide bond (*14*). The gene that encodes β-lactamase has been sequenced (*14*), the three-dimensional structure of a homologous class A β-lactamase has been solved (*15*), and a simple spectrophotometric assay exists for enzyme activity (*16*). The enzyme inactivates β-lactam antibiotics (penicillins and cephalosporins) by hydrolyzing the β-lactam amide bond. The reaction proceeds through a two-step mechanism involving nucleophilic attack of Ser^{70} to form an acyl-enzyme intermediate, which is then hydrolyzed to yield the corresponding acid and free enzyme (*17–19*).

As the first target for mutagenesis we chose Phe^{66}, which is conserved in four class A β-lactamases (*20*), since a number of L-phenylalanine analogues are easily synthesized and phenylalanine does not require additional side chain protection in the chemical aminoacylation step. A 2.5 Å crystal structure of the *Staphyloccus aureus* enzyme (33 percent homology with the *E. coli* enzyme) localizes the residue to an extended loop between a buried β sheet and an α-helical domain containing the active site (*15*). The structural importance of this residue was confirmed by constructing the Phe^{66} → Ala (pF66A) and Phe^{66} → Tyr (pF66Y) mutants (Fig. 1), both of which yielded little activity in crude cell extracts (*21, 22*). Attempts at purification resulted in loss of all activity for the F66A mutant, whereas the F66Y mutant was purified in low yield and characterized. The Michaelis constant (K_m) of the in vivo synthesized F66Y mutant for nitrocefin was identical to that of the wild-type enzyme, whereas the catalytic rate constant k_{cat} was 16 percent of that for wild-type enzyme.

In vivo and in vitro synthesis of β-lactamase was carried out with the plasmid pSG7 (Fig. 1), which was designed with the following considerations: RTEM β-lactamase is synthesized in vivo with a 23–amino acid leader sequence that is clipped off during translocation across the inner membrane to yield the fully active enzyme. In order to express active enzyme in vitro, we used a truncated gene for β-lactamase (*23*) in which a 63-bp deletion corresponding to a 21–amino acid deletion in the leader sequence is sufficient for direct expression of active enzyme. The truncated gene was placed under the transcriptional control of the strong hybrid *tac* promoter (*24*), as it has been demonstrated that the amount of protein synthesized in an in vitro translation system is proportional to the amount of messenger RNA (mRNA) added (*25*). To this end, the truncated gene was also placed under control of the ϕ10 promoter (*26*) from bacteriophage T7 with the intent of supplementing the reaction with T7 RNA polymerase, which synthesizes RNA at a rate ten times that of the *E. coli* polymerase (*27*) [plasmid pSG1 (*28*)]. The

kanamycin resistance gene from Tn*903* (*29*), cloned in the opposite orientation from the *tac* and T7 promoters, provides a selectable marker for these plasmids.

Protein synthesis was carried out in vitro in order to simplify addition of the aminoacylated suppressor tRNA to the translational machinery. The coupled *E. coli* system developed by Zubay (*30*), with some modifications by Collins (*31*) and Pratt (*22*), was used with little further modification except for lowering the *p*H of the system from 8.2 to 7.4 in order to better stabilize the base-labile acyl linkage of the added aminoacylated suppressor (Fig. 2).

Yields of active β-lactamase synthesized in this system primed with pSG7 typically ranged from 30 to 45 μg per milliliter of reaction mixture, based on the nitrocefin hydrolysis assay (Fig. 2 and Table 1). This yield corresponds to 23 to 33 copies of active enzyme per copy of gene and represents an 11-fold increase in synthesized enzyme compared with that directed from the wild-type Ap^r promoter of the pBR322 derivative pSG1. (The amount of overproduction in vivo, that is, JM101/pSG7 versus JM101/pSG1, is also 11-fold, based on the specific activity of crude cell extracts.) Addition of T7 RNA polymerase (to a final concentration of 49,000U/ml) to reactions primed with the T7 promoter plasmid pSG1 yielded levels of active enzyme that were comparable to the levels produced in reactions primed with pSG7. [In vitro protein synthesis of 434 repressor expressed behind the strong *tac* promoter afforded greater than 150 copies of protein per copy of gene.] The β-lactamase that was produced in vitro was purified to homogeneity by a sequence of ammonium sulfate precipitation, chromatofocusing, and anion exchange chromatography (Fig. 2). The protein was determined to be homogeneous by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and had a k_{cat} and K_m for nitrocefin identical to that of enzyme produced in vivo. All suppression work was performed with the pSG7 derivative pF66*am* (Fig. 1), which carries the $Phe^{66} \rightarrow$ TAG mutation.

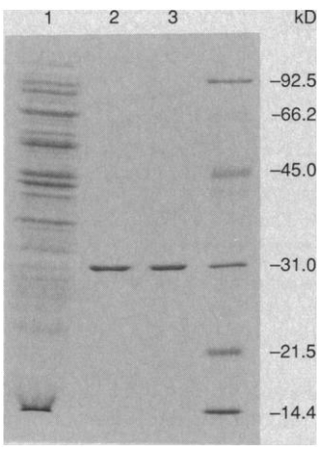


Fig. 2. In vitro synthesis and purification of truncated β-lactamase. In vitro reactions contained, per milliliter: 56.4 μmol tris-acetate, *p*H 7.4; 1.76 μmol of dithiothreitol; 1.22 μmol of ATP (Na), *p*H 7.0; 0.85 μmol each of GTP (Na), CTP (Na), and UTP (Na), *p*H 7.0, 27 μmol sodium phosphoenol pyruvate, *p*H 7.0; 0.35 μmol each of the 20 amino acids; 19 mg of polyethylene glycol 8000; 35 μg of folinic acid; 27 μg of pyridoxine · HCl, 27 μg of NADP, 27 μg of FAD, 11 μg of *p*-aminobenzoic acid, 170 μg of *E. coli* tRNA; 36 μmol of ammonium acetate, 72 μmol of potassium acetate, 9.7 μmol of calcium acetate, and 10 to 14 μmol of magnesium acetate. [The system is highly sensitive to magnesium ion concentration, which was optimized (with a β-lactamase activity assay) for each preparation of S-30, plasmid, and suppressor.] Plasmid DNA (100 μg) (*54*) was preincubated at 37°C for 2 minutes with the above components, after which suppressor tRNA (167 μg/ml of desalted and lyophilized tRNA suspended in 1 m*M* potassium acetate, *p*H 4.5, immediately prior to use) and S-30 (285 μl/ml) (*22*) were added. Reactions were incubated at 37°C for 1 hour on a rotary shaker (200 rpm), cooled to 0°C, and centrifuged. Enzyme was purified from a 1-ml pSG7-primed reaction as follows. After incubation, the reaction mixture was centrifuged and the supernatant was treated with 25 μg each deoxyribonuclease I and RNase A at 37°C for 10 minutes. After cooling to 0°C, 500 mg of ammonium sulfate was added slowly with vortexing. Precipitated proteins were collected by centrifugation, suspended in 1 ml of 25 m*M* bis-tris-HCl, *p*H 6.6 (buffer P), and desalted on an FPLC fast desalting column (Pharmacia) equilibrated in the same buffer. Active fractions were pooled (2 ml) and applied to a chromatofocusing column (Pharmacia Mono P) equilibrated with buffer P. The column was washed until the absorbance at 280 nm (A_{280}) was less than 0.05, and β-lactamase was eluted with a *p*H gradient created by washing the column (flow rate 0.5 ml/min) with tenfold-diluted Polybuffer 74-HCl, *p*H 4.6. Active fractions were pooled (1 ml), diluted tenfold with 20 m*M* tris-HCl, *p*H 7.5 (buffer A), and chromatographed on an FPLC Mono Q anion exchange column (Pharmacia). Enzyme was eluted with a gradient of 0 to 12 percent B (B = A + 1*M* KCl) in 20 minutes at a flow rate of 1 ml/min. A typical yield was 5 μg (19 percent) of purified enzyme, starting from 27 μg of enzyme in the crude reaction. Samples (2 μg per band) were separated on a 12.5 percent SDS-polyacrylamide gel (*56*), which was subsequently stained with Coomassie Brilliant Blue R-250; (lane 1) crude in vitro reaction; (lane 2) purified β-lactamase synthesized in vitro; and (lane 3) purified β-lactamase synthesized in vivo (JM101/pSG7).

Suppressor tRNA generation and characterization. The suppressor tRNA used to deliver the unnatural amino acid to the growing peptide chain on the ribosome must meet two criteria: (i) it must efficiently insert the amino acid in response to the UAG message, and (ii) it must be neither acylated nor deacylated by any of the *E. coli* aminoacyl-tRNA synthetases present in the in vitro transcription-translation system. The first condition is necessary for producing quantities of protein that can be purified and further studied, and the second condition is required to ensure that only the desired unnatural amino acid and not one or more of the 20 natural amino acids in the in vitro reaction are inserted into the protein (*32*). An amber suppressor tRNA derived from yeast $tRNA^{Phe}$ (*33*) was expected to meet these requirements on the basis of the following observations. Yeast $tRNA^{Phe}_{CUA}$, in which residues 34 to 37 of yeast $tRNA^{Phe}$ are replaced by 5′-CUAA-3′, is expected to be an efficient suppressor based on Yarus' extended anticodon loop hypothesis (*34*). In addition, Bruce and co-workers (*9*) demonstrated that yeast $tRNA^{Phe}_{CUA}$ was efficient in translating UAG codons in a mammalian protein synthesizing system (although being somewhat less efficient in a wheat germ system). Kwok and Wong (*35*) have shown that *E. coli* phenylalanyl-tRNA synthetase (PRS) aminoacylates yeast $tRNA^{Phe}$ less than 1 percent as well as it acylates *E. coli* $tRNA^{Phe}$.

Yeast $tRNA^{Phe}_{CUA}$ was prepared in milligram quantities according

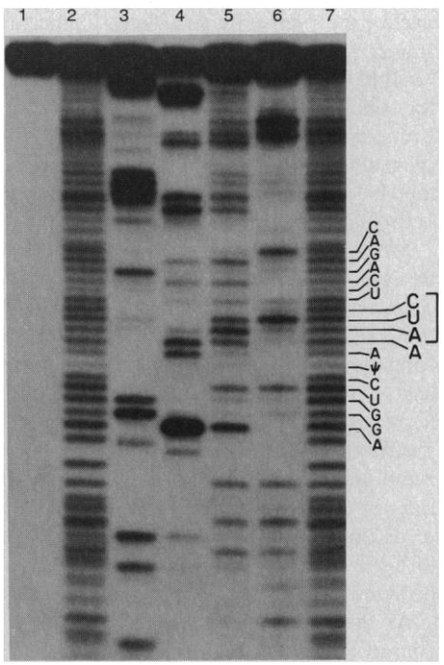


Fig. 3. $tRNA^{Phe}_{CUA}$-(-CA) was 3′ end-labeled with [5′-^{32}P]pCp, and the sequence was determined by the enzymatic method (*57*). The products of the digestion reactions were loaded onto a 10 percent denaturing polyacrylamide gel. An autoradiogram of the sequencing gel is shown: (lane 1) no enzyme; (lanes 2 and 7) $^{-}$OH digest; (lane 3) RNase T1 (G-specific); (lane 4) RNase U2 (A-specific); (lane 5) RNase Phy M (U- and A-specific); and (lane 6) RNase *Bacillus cereus* (U- and C-specific). The sequence of the anticodon stem and loop is shown, and the site of CUAA incorporation is indicated by larger letters. The anticodon is indicated by the bracket. The $tRNA^{Phe}_{CUA}$-(-CA) was either purified by preparative gel electrophoresis and used in chemical misacylation reactions, or treated with nucleotidyl transferase (*39*), gel-purified, and used in misacylation reactions with yeast PRS.

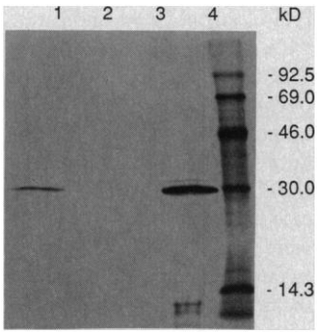


Fig. 4. Test of acylated and nonacylated suppressor in vitro. Reactions (30 μl) were carried out as described in Fig. 2, cooled to 0°C, and centrifuged. A portion (3 μl) of each supernatant was denatured and loaded onto a 12.5 percent SDS-polyacrylamide gel (*56*), which was dried and autoradiographed after electrophoresis: (lane 1) reaction primed with pSG7 (truncated β-lactamase); (lane 2) reaction primed with pF66*am*, with no added suppressor; and (lane 3) reaction primed with pF66*am*, with non-acylated suppressor (5 μg) added. Lanes 1, 2, and 3 were supplemented with [^{3}H]Phe (Amersham) to a final specific activity of 190 Ci/mol; Lane 4: reaction primed with pF66*am* and 5 μg of suppressor that had been acylated enzymatically (*40*) with [^{3}H]Phe (specific activity 9.4 Ci/mmol Phe-tRNA). The β-lactamase activity in the supernatants of these reactions was measured with the nitrocefin hydrolysis assay (*16*). The activities (in μg/ml) were: reaction 1, 44.6; reactions 2 and 3, 0; and reaction 4, 6.7.

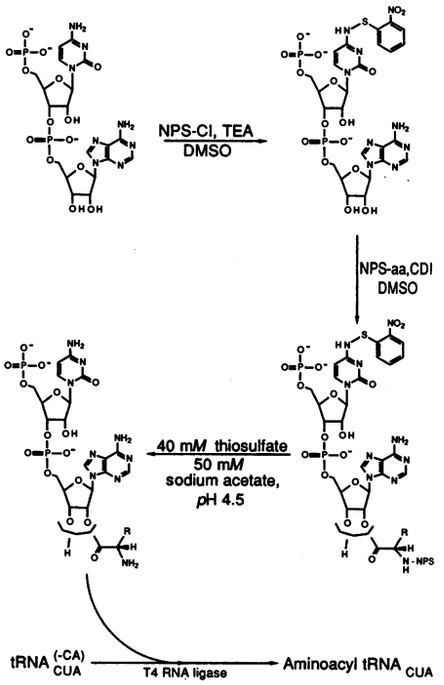


Fig. 5. Chemical aminoacylation of the dinucleotide pCpA (*58*). *o*-Nitrophenylsulfenyl chloride (1.8 mmol) and triethylamine (TEA, 1.8 mmol) were added over 6 hours to pCpA (285 μmol) dissolved in dimethyl sulfoxide (DMSO) (68 ml). The reaction was quenched by addition of 50 m*M* ammonium acetate, *p*H 5 (100 ml), and the solvent was removed under vacuum. Purification by reversed-phase HPLC (*59*) yielded NPS-pCpA in 65 percent yield and recovered pCpA in 22 percent yield. NPS-pCpA was desalted by reversed-phase HPLC (*60*) and passed through a Dowex column (Li^+ form). *o*-Nitrophenylsulfenylphenylalanine (Nps-aa, 74 μmol) and *N*,*N*′-carbonyldiimidazole (CDI, 83 μmol) were stirred under nitrogen in anhydrous dimethyl sulfoxide (320 μl) for 30 minutes. The solution was then added to the lithium salt of NPS-pCpA (15 μmol, dried by repeated coevaporation with toluene). The reaction was stirred under nitrogen at 50°C for 8 hours and was then quenched at 0°C by addition of 50 m*M* ammonium acetate, *p*H 5 (2 ml). Lyophilization followed by reversed-phase HPLC (*61*) provided the desired product in 16 percent yield with 38 percent starting material being recovered. After lyophilization, the product (2.4 μmol) was dissolved in 40 m*M* sodium thiosulfate, 50 m*M* sodium acetate, *p*H 4.5 (2 ml), and stirred for 1 hour. Reversed-phase HPLC (*62*) afforded the deprotected acyl pCpA in 81 percent yield. All products were characterized by ultraviolet spectroscopy and by conventional and two-dimensional nuclear magnetic resonance.

to the anticodon-loop replacement procedure of Bruce and Uhlenbeck (*33, 36*) (Fig. 3). This procedure involved removal of the three anticodon nucleotides G-34, A-35, and A-36, as well as the modified nucleotide Y-37 from the anticodon loop of yeast $tRNA^{Phe}$. The four excised nucleotides were then replaced with a chemically synthesized CpUpApA that included the anticodon sequence required for an amber suppressor tRNA. The suppressor produced by this method is lacking the 3′ terminal pCpA aminoacyl acceptor stem. These nucleotides can be replaced through the use of the tRNA repair enzyme nucleotidyl transferase (*39*) to yield a full-length yeast $tRNA^{Phe}_{CUA}$. The suppressor tRNA can be aminoacylated in vitro with [^{3}H]phenylalanine to 30 to 35 percent (based on radioactivity incorporated into purified [^{3}H]Phe-$tRNA^{Phe}_{CUA}$) with the use of a large excess of yeast PRS (*40*). Under similar reaction conditions wild-type yeast $tRNA^{Phe}$ acylates to 40 to 45 percent with yeast PRS. Attempts at separating acylated from non-acylated tRNA's by BD-cellulose chromatography (*6, 11*) resulted in poor separation and unacceptably low yields of acylated tRNA. The mixture of acylated and non-acylated tRNA's was therefore used directly in in vitro protein synthesis reactions. We have attempted to misacylate $tRNA^{Phe}_{CUA}$ with several analogs of Phe with yeast PRS; however, these experiments were unsuccessful under variations in *p*H, concentration of buffer or salt or both, and concentration of organic solvents.

Importantly, yeast $tRNA^{Phe}_{CUA}$ is not recognized by the *E. coli* aminoacyl-tRNA synthetases present in our in vitro system (Fig. 4). An in vitro reaction primed with pF66*am* and non-acylated suppressor, in the presence of [^{3}H]Phe, resulted in no β-lactamase activity and no radioactive band of the correct molecular weight when analyzed on a denaturing polyacrylamide gel. A reaction primed with pF66*am* and [^{3}H]Phe-$tRNA^{Phe}_{CUA}$ resulted in β-lactamase activity and a radioactive band of the correct molecular weight for β-lactamase. At this concentration of Phe-$tRNA^{Phe}_{CUA}$, the level of in vitro β-lactamase synthesis from pF66*am* was 15 to 20 percent compared with that for pSG7. These results demonstrated that yeast $tRNA^{Phe}_{CUA}$ meets the design criteria outlined above: the tRNA is not enzymatically aminoacylated or deacylated, and it inserts an acylated amino acid in response to UAG.

We have also produced a runoff transcript tRNA (*41*) corresponding to the sequence of yeast $tRNA^{Phe}_{CUA}$(-CA), which has been purified and ligated to pCpA-Phe (*42*). Addition of this aminoacylated tRNA to in vitro protein synthetases reactions afforded 50 to 65 percent of the β-lactamase activity observed for reactions in which the same amounts of Phe-$tRNA^{Phe}_{CUA}$ derived from anticodon loop replacement were used (*42*). This level of translational efficiency for the runoff suppressor compares favorably to data for a $tRNA^{Gly}$ constructed in a similar fashion (*43*).

Chemical aminoacylation. As noted above, enzymatic misacylation by the aminoacyl-tRNA synthetases is not a general method because of the high specificity of these enzymes. Chemical misacylation, however, should be generalizable to any amino acid–like structure. Direct chemical acylation of an intact tRNA is not practical because of the large number of reactive sites in the macromolecule. Hecht and co-workers (*11*) simplified this problem by chemically acylating the dinucleotide pCpA and enzymatically ligating it to the 3′ terminus of a truncated tRNA [tRNA(-CA)] with T4 RNA ligase to afford an aminoacyl tRNA. This approach, although successful, suffered two major drawbacks: (i) the α-amino protecting group was not removed, which restricted the aminoacyl tRNA to act only as a P-site donor, and (ii) the chemical acylation yield was quite low.

The general strategy for chemical acylation of pCpA involves carboxyl activation of an N-blocked amino acid which is then coupled through an ester linkage to the diol of the terminal adenosine (the 2′ and 3′ acyl groups rapidly interconvert in aqueous solution). Aminoacylation is complicated by preferential acylation of the exocyclic amino group of cytidine and 2′,3′-diacylation of adenosine. The α-amino protecting group greatly increases the stability of the aminoacyl ester linkage to hydrolysis and prevents polymerization during carboxyl activation (*44*). However, the protecting group must be removed if the acylated tRNA is to function

as an A-site donor. Brunner (*6*) recently showed that α-amino protected aminoacyl pCpA can be deprotected and ligated to tRNA(-CA) with acceptable levels of hydrolysis of the aminoacyl ester linkage.

The scheme for aminoacylation of $tRNA^{Phe}_{CUA}$ is outlined in Fig. 5. We used a minimal protection scheme in which only the exocyclic amine of cytidine was protected by *o*-nitrophenyl sulfenyl chloride (NPS-Cl). The α-amino group of the amino acid was also protected with the NPS group. NPS-pCpA was acylated with N-blocked Phe with the use of *N*, *N*′-carbonyldiimidazole as the activating agent. The NPS protecting groups were removed in high yield from cytidine and the amino acid with aqueous thiosulfate (*45*). The acylation-deprotection was carried out in 14 percent overall yield (*46*), which compares favorably with the 3 to 4 percent yields of Hecht and Brunner (*6, 11*). However, Chladek (*47*) has recently reported aminoacylation of 5′-CpCpA in 26 percent overall yield through an alternate strategy.

Fully deprotected pCpA-Phe was ligated directly to $tRNA^{Phe}_{CUA}$-(-CA) with T4 RNA ligase (*48*) (note that the truncated suppressor tRNA is generated directly by the anticodon loop replacement method) (*49*). The yield of Phe-$tRNA^{Phe}_{CUA}$ is 35 percent based on analysis of [^{3}H]Phe incorporation into the purified suppressor (*48*) [gel electrophoresis indicates 80 to 90 percent of the $tRNA^{Phe}_{CUA}$-(-CA) is converted to material with the same mobility as $tRNA^{Phe}_{CUA}$]. We used this procedure to aminoacylate $tRNA^{Phe}_{CUA}$(-CA) with D-phenylalanine (D-Phe), (*S*)-*p*-nitrophenylalanine (*p*-NO_2Phe), (*S*)-homophenylalanine (2-amino-4-phenylbutanoic acid, HPhe), (*S*)-*p*-fluorophenylalanine (*p*-FPhe), (*S*)-3-amino-2-benzylpropionic acid (ABPA), and (*S*)-2-hydroxy-3-phenylpropionic acid (PLA) (in this case no α-hydroxyl protection was used). These aminoacyl tRNA's were used in in vitro protein synthesis to synthesize mutant β-lactamases (see below). Current efforts to optimize aminoacylation include the use of acid-labile protecting groups and protecting groups that can be removed by hydrogenation, as well as an investigation of the use of nonselective lipases for the aminoacylation of unprotected RNA. Protecting groups that could be removed by hydrogenolysis or acid treatment would also simplify protection of unnatural amino acid side chains. [Acylation of NPS-pCpA with N-Cb_z-Phe followed by deprotection with aqueous thiosulfate and catalytic hydrogenation affords pCpA-Phe in 35 percent isolated yield.]

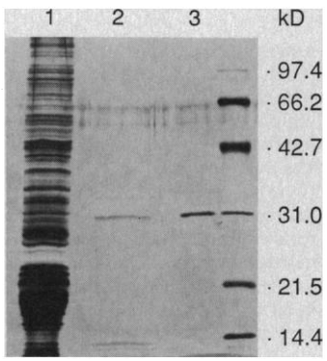


Fig. 6. Purification of Phe^{66} β-lactamase synthesized according to scheme 1. β-lactamase was purified from a 900-μl pF66*am*-primed reaction that had been supplemented with 150 μg of chemically acylated $tRNA^{Phe}_{CUA}$ as described in Fig. 2. Typical yields were 0.3 to 0.7 μg (7 to 15 percent) of purified enzyme, starting from 4.5 μg in the crude reaction. Samples (50 to 200 ng per band) were analyzed on a 12.5 percent SDS-polyacrylamide gel (*56*), which was subsequently silver stained. The diffuse bands at relative molecular masses of 67,000 and 60,000 are artifacts commonly observed during high-sensitivity silver staining (*63*): (lane 1) crude in vitro reaction; (lane 2) purified in vitro β-lactamase; and (lane 3) purified in vivo β-lactamase (JM101/pSG7).

In vitro suppression. In vitro reactions primed with pF66*am* and supplemented with suppressor that had been enzymatically acylated with [^{3}H]Phe (30 percent acylated), to a final concentration of 167 μg/ml, yielded 5.5 to 7.5 μg/ml of active β-lactamase, which represents 15 to 20 percent suppression efficiency (Fig. 6). A suppressor that had been chemically acylated with pCpA-Phe (35 percent acylated) resulted in a yield of 2.8 to 7.5 μg/ml. This yield is sufficient for purification of the enzyme to near homogeneity from a 1-ml reaction in 7 to 15 percent overall yield (Fig. 6) with a sequence of ammonium sulfate precipitation, chromatofocusing, and anion exchange chromatography. The k_{cat} and K_m for the purified β-lactamase were identical to those of the wild-type enzyme produced in vitro (Table 1). Site-specific insertion of [^{3}H]Phe by [^{3}H]Phe-$tRNA^{Phe}_{CUA}$ into β-lactamase was verified by peptide mapping experiments. There are 27 putative trypsin cleavage sites, and 5 Phe residues in RTEM β-lactamase (*14*). These 5 Phe residues are distributed in 4 of the 28 tryptic fragments, with one 8-residue peptide containing both Phe^{66} and Phe^{72}. We synthesized β-lactamase in vitro from pSG7 in the presence of added [^{3}H]Phe. The purified labeled enzyme was digested with trypsin, and the frag-

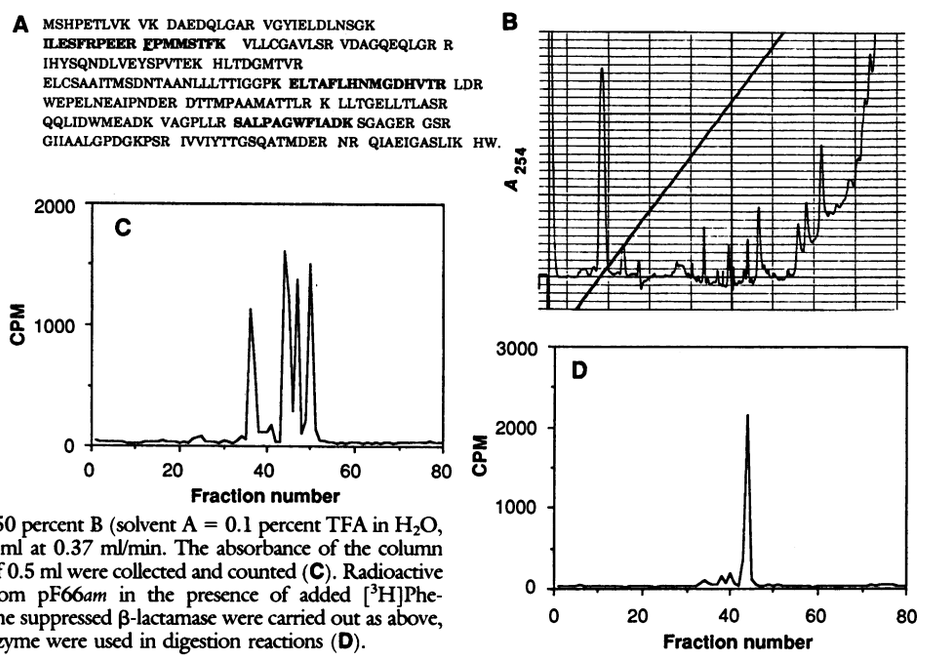


Fig. 7. Peptide mapping of wild-type and suppressed β-lactamase. Wild-type β-lactamase was uniformly labeled with [^{3}H]Phe by in vitro protein synthesis from pSG7 in the presence of added [^{3}H]Phe. Nonlabeled β-lactamase was added to the products of the in vitro synthesis prior to purification of the enzyme by gel filtration on Sephadex G-75 (Pharmacia) and chromatofocusing chromatography as described in Fig. 2. Trypsin digestion reactions (400 μl total volume) contained the following: 15,000 counts per minute (CPM) purified, uniformly labeled β-lactamase, 40 μg of nonlabeled β-lactamase, 2*M* urea, 0.1 m*M* $CaCl_2$, 200 m*M* NH_4HCO_3 (*p*H 8.5), and 1.5 units of trypsin (Cooper Biomedical, TPCK-treated, 251 U/mg). The reactions were incubated in the dark at 25°C for 16 hours and then quenched by addition of glacial acetic acid to 10 percent (v/v). (**A**) The sequence of β-lactamase from pSG7. Trypsin cleavage sites are indicated by spaces, peptides containing Phe are indicated by bold type, and Phe^{66} is underlined (*64*). The tryptic peptides were separated with a Pharmacia Pep RPC 5/5 column. Gradient conditions were 0 to 50 percent B (solvent A = 0.1 percent TFA in H_2O, solvent B = 0.08 percent TFA in CH_3CN) over 40 ml at 0.37 ml/min. The absorbance of the column effluent was monitored at 254 nm (**B**), and fractions of 0.5 ml were collected and counted (**C**). Radioactive suppressed β-lactamase was synthesized in vitro from pF66*am* in the presence of added [^{3}H]Phe-$tRNA^{Phe}_{CUA}$. The purification and trypsin digestion of the suppressed β-lactamase were carried out as above, except that only 6000 CPM of labeled suppressed enzyme were used in digestion reactions (**D**).

ments were separated by reversed-phase fast protein liquid chromatography (FPLC) (Fig. 7). Four discrete radioactive peaks were observed, in agreement with the locations of [^{3}H]Phe in RTEM β-lactamase (*14, 17*). The peak that eluted in fractions 44 and 45 contained twice as many counts as the other three peaks and was assigned as the peptide containing Phe^{66} and Phe^{72}. A similar analysis of tryptic peptides derived from β-lactamase synthesized in vitro from pF66*am* in the presence of added [^{3}H]Phe-tRNA$^{Phe}_{CUA}$ showed one radioactive peak. The presence of radioactivity in fraction 44 in both the wild-type (pSG7) and suppressed (pSF66*am*) experiments, taken together with the observation that in the wild-type experiments this peak contains twice as much radioactivity as the others, strongly suggests that in the suppressed experiment [^{3}H]Phe is inserted only at the target site (Phe^{66}).

The Phe analogues D-Phe, *p*-FPhe, *p*-NO_2Phe, HPhe, ABPA, and PLA were each loaded onto suppressor tRNA as described above. In vitro protein synthesis reactions carried out in the presence of [^{35}S]Met resulted in similar levels of radioactivity incorporated into trichloroacetic acid (TCA)–precipitable material for the *p*-FPhe, *p*-NO_2Phe, and HPhe reactions. Kinetic analyses of the β-lactamases synthesized in these reactions demonstrated similar K_m's but different k_{cat}'s (Table 1). Direct quantitation of the purified *p*-NO_2Phe and HPhe mutants was not possible, as both mutants lost activity during purification attempts. Thus we used [^{35}S]Met incorporation and TCA precipitation to quantitate all of the mutants for the purpose of direct comparison. Experiments with D-Phe, PLA, and ABPA resulted in no detectable β-lactamase activity or protein synthesis.

Properties of unnatural amino acid mutants. These results demonstrate that for phenylalanine, analogues that differ in both steric and electronic properties can be substituted into proteins. Replacement of Phe^{66} by Tyr, which differs from Phe both sterically (4-OH group) and electronically (OH is a good π-electron donor), leads to an approximate twofold decrease in k_{cat} with little effect on K_m. Similarly, replacement of Phe^{66} by *p*-NO_2Phe, which again differs both sterically (4-NO_2 group) and electronically (NO_2 is a good π-electron acceptor), leads to an approximate twofold decrease in k_{cat} with little effect on K_m. On the other hand, replacement of Phe^{66} by *p*-FPhe, which is both sterically and electronically similar to Phe, leads to a slight increase in k_{cat} with no effect on K_m. Replacement of Phe^{66} with HPhe, which is electronically identical to Phe but substantially different sterically, leads to an approximate sixfold decrease in k_{cat} and an increase in K_m. Both the HPhe and *p*-NO_2Phe mutants, which correspond to the greatest steric perturbation, are unstable and presumably unfold and proteolyze during purification attempts.

Attempts to alter the protein backbone by replacing the amide linkage with an ester linkage (PLA), adding an additional methylene group into the backbone (ABPA), or changing the stereochemistry of the α-carbon (D-Phe) lead to no detectable synthesized protein or enzymatic activity. It is not clear at present whether these results stem from impaired protein folding or stability (Phe^{66} is adjacent to a Pro residue) or from inability of these amino acids to function as ribosomal A-site acceptors. It has been reported that PLA is incorporated into the amino-terminal position of polyphenylalanine (*50*) and that *N*-acetyl-D-phenylalanine functions poorly as a P-site donor in response to a poly(U) message (7). Yamane *et al.* report that the dissociation constant K_d for the D-Tyr-tRNA–EF-Tu ternary complex is 25-fold greater than for the L-Tyr-tRNA–EF-Tu ternary complex (*51*). The same level of stereochemical selectivity for ternary complex formation between EF-Tu and D-Phe-tRNA$^{Phe}_{CUA}$ would result in substantial hydrolysis of the aminoacyl ester during in vitro reactions as well as poor EF-Tu mediated binding of the aminoacylated tRNA to the ribosome.

Further interpretation of the properties of the mutant enzymes awaits the availability of an x-ray crystal structure of RTEM β-lactamase. We are currently generating mutants at Ser^{70} of β-lactamase and in the DNA binding domain of 434 repressor. These experiments should begin to define the potential and generality of this methodology for probing protein structure and function.

The methodology described above has made possible the site-specific substitution of unnatural amino acids with novel steric and electronic properties into proteins. Sufficient protein can be purified to characterize the catalytic constants and specificity of the mutants, to carry out limited mechanistic and mapping studies, and to probe protein structure with techniques such as ESR and fluorescence spectroscopy. Improvements in in vitro protein synthesis, methods for tRNA generation, and tRNA aminoacylation chemistry should make it possible to produce milligram quantities of mutant proteins with this strategy.

REFERENCES AND NOTES

1. R. B. Leatherbarrow and A. R. Fersht, *Protein Eng.* **1**, 7 (1986); D. A. Estell *et al.*, *Science* **233**, 659 (1986); J. R. Knowles, *ibid.* **236**, 1252 (1987).
2. K. Hofman and H. Bohn, *J. Am. Chem. Soc.* **88**, 5914 (1966).
3. F. Borras and R. E. Offord, *Nature* **227**, 716 (1970); R. W. Sealock and M. Laskowski, *Biochemistry* **8**, 3703 (1969); K. Inouye *et al.*, *J. Am. Chem. Soc.* **101**, 752 (1979).
4. K. E. Neet and D. E. Koshland, *Proc. Natl. Acad. Sci. U.S.A.* **56**, 1606 (1966); L. Polgar and M. L. Bender, *J. Am. Chem. Soc.* **88**, 3153 (1966); E. T. Kaiser and D. S. Lawrence, *Science* **226**, 505 (1984); S. L. Mayo *et al.*, *ibid.* **233**, 948 (1986).
5. U. C. Kreig, P. Walter, A. E. Johnson, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 8604 (1986); M. Wiedmann *et al.*, *Nature* **328**, 830 (1987); A. Johnson *et al.*, *Biochemistry* **15**, 569 (1976).
6. G. Baldini, B. Martoglio, A. Schachenmann, C. Zugliani, J. Brunner, *Biochemistry* **27**, 7951 (1988).
7. J. R. Roesser, M. S. Chorghade, S. M. Hecht, *ibid.* **25**, 6361 (1986); T. G. Heckler *et al.*, *J. Biol. Chem.* **258**, 4492 (1983).
8. J. H. Miller, D. Ganem, P. Lu, A. Schmitz, *J. Mol. Biol.* **109**, 275 (1977); L. Bossi and J. R. Roth, *Nature* **286**, 123 (1980); D. A. Steege, in *Biological Regulation and Development*, R. Goldberger, Ed. (Plenum, New York, 1978), vol. 1.
9. A. Bruce *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7127 (1982).
10. F. Chapeville *et al.*, *ibid.* **48**, 1086 (1962); K. Jacobsen, *Cold Spring Harbor Symp. Quant. Biol.* **31**, 719 (1966).
11. T. G. Heckler *et al.*, *Tetrahedron* **40**, 87 (1984); T. G. Heckler *et al.*, *Biochemistry* **23**, 1478 (1984).
12. J. M. T. Hamilton-Miller and J. T. Smith, Eds., *Beta-Lactamases* (Academic Press, London, 1979).
13. M. H. Richmond, G. Jack, R. B. Sykes, *Ann. N.Y. Acad. Sci.* **182**, 243 (1971); E. P. Abraham, *J. Antibiot.* **30**, 51 (1977); J. Fisher, R. L. Charnas, J. R. Knowles, *Biochemistry* **17**, 2180 (1978).
14. J. G. Sutcliffe, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3737 (1978); R. P. Ambler and G. K. Scott, *ibid.*, p. 3732; S. Pollitt and H. Zalkin, *J. Bacteriol.* **153**, 27 (1983).
15. O. Herzberg and J. Moult, *Science* **236**, 694 (1987).
16. C. H. O'Callaghan, A. Morris, S. M. Kirby, A. H. Shingler, *Antimicrob. Agents Chemother.* **1**, 283 (1972). One nitrocefin hydrolysis unit (1 μmol of nitrocefin hydrolyzed per minute per milliliter, 0.1 m*M* nitrocefin, 50 m*M* phosphate buffer, *p*H 7) corresponds to 0.61 μg of enzyme, as determined by Bradford assay.
17. J. Fisher, J. G. Belasco, S. Khosla, J. R. Knowles, *Biochemistry* **19**, 2895 (1980); J. R. Knowles, *Acc. Chem. Res.* **18**, 97 (1985).
18. G. Dalbadie-McFarland *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6409 (1982).
19. I. S. Sigal, W. F. DeGrado, B. J. Thomas, S. R. Petteway, Jr., *J. Biol. Chem.* **259**, 5327 (1984).
20. R. P. Ambler, in (*12*), pp. 99–125.
21. All in vivo work was carried out with the *E. coli* strain JM101 (Δ*lacpro thi, supE, F'traD36, proAB, lacI^q ZΔM15*) [C. Janisch-Perron, J. Veiera, J. Messing, *Gene* **22**, 103 (1983)]. All in vitro work was done with S-30 extracts (*22*) prepared from *E. coli* strain D10 (*rna-10, relA1, spoT1, metB1*) [R. F. Gesteland, *J. Mol. Biol.* **16**, 67 (1966)].
22. J. M. Pratt, in *Transcription and Translation*, B. D. Hanes and S. J. Higgins, Eds. (IRL Press, Oxford, 1984), pp. 179–209.
23. J. T. Kadonaga *et al.*, *J. Biol. Chem.* **259**, 2149 (1984).
24. E. Amann, J. Brosius, M. Ptashne, *Gene* **25**, 167 (1983).
25. G. Reiness and G. Zubay, *Biochem. Biophys. Res. Commun.* **53**, 967 (1973).
26. S. Tabor and C. C. Richardson, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1074 (1985).
27. M. Chamberlin and T. Ryan, *Enzymes* **15**, 87 (1982).
28. C. J. Noren, unpublished results.
29. A. Oka, H. Sugisaki, M. Takanami, *J. Mol. Biol.* **147**, 217 (1981).
30. G. Zubay, *Annu. Rev. Gen.* **7**, 267 (1973).
31. J. Collins, *Gene* **6**, 29 (1979).
32. P. R. Schimmel and D. Soll, *Annu. Rev. Biochem.* **48**, 601 (1979); A. R. Fersht and M. M. Kaethner, *Biochemistry* **15**, 3342 (1976); G. L. Igoli *et al.*, *ibid.* **17**, 3459 (1978); A. R. Schreier and P. R. Schimmel, *ibid.* **11**, 1582 (1972); M. Yarus, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1915 (1972).

33. A. G. Bruce and O. C. Uhlenbeck, *Biochemistry* **21**, 3921 (1982); *ibid.*, p. 855.
34. M. Yarus, *Science* **218**, 646 (1982).
35. Y. Kwok and J. T. Wong, *Can. J. Biochem.* **58**, 213 (1980).
36. The procedure of Bruce and Uhlenbeck (*33*) was modified as follows. After the initial depurination step the tRNA-$_Y$ was recovered by ethanol precipitation and treated with aniline hydrochloride. The cleavage products were recovered by ethanol precipitation and separated on a preparative denaturing PAGE gel (8 percent, 0.015 cm by 16 cm by 42 cm; 5 mg of the crude tRNA was loaded onto each gel). The bands were stained with 0.02 percent toluidine blue, cut out and eluted twice with 10 ml of 100 m*M* sodium acetate (*p*H 4.5), 1 m*M* EDTA, and 0.1 percent SDS. The stain was removed by extractions with phenol and $CHCl_3$, and the tRNA half-molecules were recovered by ethanol precipitation. For the partial nuclease digestion, the concentration of the ribonuclease A (RNase A) was increased to 2 μg/ml. After recovery of the digested half-molecules by ethanol precipitation, the pellet was resuspended in sterile water and extracted with phenol, phenol:$CHCl_3$ (1:1), and $CHCl_3$, and was then reprecipitated with ethanol. The ribonucleotide tetramer CpUpApA was synthesized by sequential phosphotriester coupling of protected nucleosides (*37*). The fully deprotected CUAA was ligated onto the 3′ tRNA half-molecule with T4 RNA ligase (Takara Shuzo). The conditions for the ligation were 50 μ*M* adenosine triphosphate (ATP), 19 μ*M* tRNA (both half-molecules were present in the reaction), 820 μ*M* CUAA, and T4 RNA ligase at 50 U/ml. Ligation reactions were typically carried out on 5 mg of the RNase A–digested tRNA in a reaction volume of 10 ml. The kinase treatment was carried out with 4 μ*M* tRNA, 120 μ*M* ATP, and 50 U/ml of T4 polynucleotide kinase (*38*). The final ligation was done with 25 U/ml of T4 RNA ligase.
37. S. S. Jones *et al.*, *Tetrahedron* **36**, 3015 (1980); J. H. Van Boom and C. Wreesman, in *Oligonucleotide Synthesis*, M. Gait, Ed. (IRL Press, Washington, DC, 1984).
38. C. C. Richardson, *Proc. Natl. Acad. Sci. U.S.A.* **54**, 158 (1965); C. A. Midgley and N. E. Murray, *EMBO J.* **4**, 2695 (1985).
39. H. Cudney and M. P. Deutscher, *J. Biol. Chem.* **261**, 6450 (1986).
40. Enzymatic misacylation reactions (300 μl total volume) contained the following: 4 μ*M* $tRNA^{Phe}_{CUA}$ (30 μg, which had been desalted and lyophilized after gel purification), 80 μ*M* Phe, 40 m*M* tris-HCl (*p*H 8.5), 15 m*M* $MgCl_2$, bovine serum albumin (BSA) at 45 μg/ml, 3.3 m*M* dithiothreitol (DTT), 2 m*M* ATP, and 22 units of yeast PRS [where 1 unit of activity incorporates 100 pmol of phenylalanine in 2 minutes at 37°C under the following conditions: 2 μ*M* $tRNA^{Phe}$ (Boehringer Mannheim), 2 m*M* ATP, 3.3 m*M* DTT, 8.1 μ*M* Phe, 40 m*M* Na salt of Hepes (*p*H 7.4), 15 m*M* $MgCl_2$, 25 m*M* KCl, and BSA at 50 μg/ml]. The reaction mixture was incubated at 37°C for 3 minutes and was then quenched by addition of 2.5*M* sodium acetate (*p*H 4.5) to 10 percent (v/v). The quenched reaction was immediately extracted with phenol (equilibrated with 0.25*M* sodium acetate, *p*H 4.5), phenol:$CHCl_3$ (1:1), $CHCl_3$, and was then precipitated with ethanol. The extraction and precipitation were repeated two more times. The tRNA was then desalted on a Pharmacia fast desalting column and lyophilized. The lyophilized mixture of acylated and nonacylated tRNA was stored at −80°C until immediately prior to its use in in vitro protein synthesis reactions.
41. J. Sampson and O. C. Uhlenbeck, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 1033 (1988).
42. D. Suich and K. Noren, unpublished results.
43. T. Samuelsson *et al.*, *J. Biol. Chem.* **263**, 1392 (1988).
44. F. Schubert and M. Pinck, *Biochimie* **56**, 383 (1974).
45. Y. Lapidot, D. Elat, S. Rappoport, N. deGroot, *Biochem. Biophys. Res. Commun.* **38**, 559 (1970); F. Heikkilä, N. Balgobin, J. Chattopadhyaya, *Acta Chem. Scand.* **B37**, 857 (1983).
46. Unpublished results in our laboratory group indicate that 2′,3′-diacylation is the major factor limiting acylation yields in model compounds.
47. E. Happ, C. Scalfi-Happ, S. Chladek, *J. Org. Chem.* **52**, 5387 (1987).
48. Chemical acylation reactions (80 μl total volume) contained the following: 600 μ*M* pCpA-Phe (40 μg), 10 μ*M* $tRNA^{Phe}_{CUA}$ (20 μg, which had been desalted and lyophilized after gel purification), 55 m*M* Hepes (*p*H 7.5), 250 μ*M* ATP, 15 m*M* $MgCl_2$, BSA at 20 mg/ml, dimethyl sulfoxide (DMSO) [to 10 percent (v/v)], and 200 units T4 RNA ligase. The reaction mixture was incubated at 37°C for 12 minutes, quenched by addition of 2.5*M* sodium acetate (*p*H 4.5) to 10 percent (v/v), and treated as described in (*40*), but with only one round of extraction and precipitation.
49. The di-NPS–protected aminoacyl pCpA was also a substrate for T4 RNA ligase, but better yields of the aminoacyl tRNA were obtained by deprotection followed by ligation rather than ligation and subsequent deprotection.
50. G. Herve and F. Chapeville, *J. Mol. Biol.* **13**, 757 (1965).
51. T. Yamane, D. L. Miller, J. J. Hopfield, *Biochemistry* **20**, 7059 (1981).
52. J. Brosius and A. Holy, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6929 (1984).
53. K. L. Nakamaye and F. Eckstein, *Nucleic Acids. Res.* **14**, 9679 (1986).
54. Plasmids were purified for in vitro protein synthesis with the use of an extension of the procedure of D. S. Holmes and M. Quigley (*55*). Plasmid DNA that had been purified in a CsCl gradient and precipitated was suspended in 2 ml of 20 m*M* potassium phosphate, *p*H 6.5, 1*M* KCl, and applied to a Nucleogen DEAE 4000-7 column equilibrated with the same buffer. Plasmid was eluted with a gradient of 50 to 70 percent B in 40 minutes; A was 20 m*M* potassium phosphate, *p*H 6.5; B was A + 2*M* KCl; the flow rate was 1.25 ml/min. Plasmid was precipitated with a water-ethanol mixture (1:4 v/v), desalted on an FPLC fast desalting column equilibrated with water, lyophilized, and suspended in 10 m*M* tris-HCl, *p*H 7.2, 1 m*M* EDTA to approximately 1 mg/ml. Typical yields of highly purified plasmid were 600 to 800 μg from 6 liters of culture.
55. D. S. Holmes and M. Quigley, *Anal. Biochem.* **114**, 193 (1981).
56. U. K. Laemmli, *Nature* **227**, 680 (1970).
57. H. Donis-Keller, *Nucleic Acids. Res.* **8**, 3133 (1980).
58. The dinucleotide pCpA was prepared by standard solution-phase phosphotriester synthesis (*37*). The fully protected molecule was 4-chlorophenyl-4-*N*-anisoyl-2′-*O*-tetrahydropyranyl-5′-*O*-[β-cyanoethyloxyphosphoryl]cytidylyl(3′-5′)-[6-*N*,2′-*O*,3′-*O*-tetrabenzoyl]adenosine.
59. High-performance liquid chromatography (HPLC) conditions: A = 5 m*M* ammonium acetate, *p*H 5, B = MeCN; gradient = 0 to 15 percent B in 60 minutes, 15 to 30 percent B in 30 minutes; flow rate = 8 ml/min; and column = Whatman Partisil 10 M-20 10/50 ODS-3.
60. HPLC conditions as in (*59*), except A = H_2O.
61. HPLC conditions as in (*59*), except A = 50 m*M* ammonium acetate and gradient = 0 to 70 percent B in 70 minutes.
62. HPLC conditions: A = 8 m*M* acetic acid, B = MeCN; gradient = 0 to 30 percent B in 45 min; flow rate = 4 ml/min; and column = Whatman Partisil 10 M9/50 ODS-3.
63. C. R. Merril, D. Goldman, M. L. Van Keuren, *Methods Enzymol.* **96**, 230 (1983).
64. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

65. We thank J. Brunner for helpful discussions regarding chemical aminoacylation, J. Knowles for plasmid pTG2*del*1, S. Tabor and C. Richardson for plasmids pT7-3 and pGP1-2, M. Deutscher for the nucleotidyl transferase expression system, and C. Midgley and N. Murray for the T4 polynucleotide kinase expression system. T4 RNA ligase was a generous gift from Takara Shuzo Corporation, and 3-amino-2-benzylpropionic acid (ABPA) was a gift from Monsanto Corporation. We especially thank H. Morimoto and P. G. Williams of the National Tritium Labelling Facility (Lawrence Berkeley Laboratory) for their expert assistance in synthesis and purification of radioactive amino acids. Supported by ONR grant N00014-86-K-0522 (mutagenesis and tRNA) and by Materials Science Division of USDOE, Office of Energy Research under contract no. DE-AC03-76SF 0098 (in vitro synthesis).

23 January 1989; accepted 6 March 1989