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Site-specific incorporation of biophysical probes into proteins

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ABSTRACT **Biophysical probes which can detect structural changes in proteins and the interaction of proteins with other macromolecules are important tools in studying protein function. Many difficulties remain, however, in introducing probes into proteins site-specifically. Here we report the successful *site-specific* incorporation of a spin-labeled, a fluorescent, and a photoactivatible amino acid into a variety of surface and internal sites in bacteriophage T4 lysozyme by using unnatural amino acid mutagenesis. In addition, we report the purification and spectral characterization of T4 lysozyme mutants containing the spin-labeled amino acid and the fluorescent amino acid. The ability to incorporate these probes site-specifically allows for novel studies of protein structure and dynamics. Moreover, this work demonstrates that the *Escherichia coli* protein biosynthetic machinery can tolerate unnatural amino acids with little resemblance to the natural amino acids.**

Probes capable of detecting local structure and dynamics in proteins are important for the study of protein folding and stability, conformational changes in proteins, and protein–protein interactions. Tryptophan can serve as an intrinsic fluorescent probe, but its utility often is limited by the presence of other tryptophans within the same protein (1). Consequently, extrinsic probes have been incorporated by chemical techniques. Spin-labels, fluorescent molecules, and photoactivatible cross-linking agents have been introduced into proteins by chemical modification of the reactive side chains of amino acids such as lysine and cysteine (2–4). Selective chemical modification of proteins, however, can be complicated by the presence of several reactive side chains in the protein and by the need to denature the protein to derivatize internal residues. In addition, semisynthetic methods have been used to site-specifically incorporate probes into proteins, but this approach currently is practical only for a limited number of relatively small proteins (5).

To avoid the difficulties associated with chemical methods, biosynthetic methods have been developed to incorporate probes into *in vitro* synthesized proteins (4, 6–8). For example, N^{ε}-(5-azido-2-nitrobenzoyl)lysine (ANB-Lys) was incorporated into preprolactin by adding ANB-Lys-tRNA$_{Lys}$ and preprolactin mRNA to an *in vitro* translation system depleted of endogenous Lys (6). A drawback to this technique is that the modified Lys, in this example, is inserted only partially and at multiple Lys positions in the protein.

Recently, a methodology has been developed that makes possible the site-specific incorporation of a wide variety of unnatural amino acids into proteins (9, 10). An amber suppressor tRNA, which is not a substrate for the endogenous aminoacyl-tRNA synthetases, is chemically modified with an unnatural amino acid. Addition of this acylated suppressor tRNA and a gene containing an amber nonsense codon at the site of interest to an *in vitro* transcription/translation system yields the desired mutant protein. Any unnatural aminoacyl-tRNA which is recognized by the *Escherichia coli* protein biosynthetic machinery—the elongation factors, ribosomes, etc.—in theory can be incorporated into the protein. This method has been used to substitute a number of amino acids with novel steric and electronic properties into proteins in studies of enzyme mechanism, protein stability, and biomolecular recognition (11–13).

To determine the feasibility of introducing biophysical probes site-specifically into proteins by using this technique, we have synthesized several spin-labeled, fluorescent, and photoactivatable amino acids and tested them for incorporation into bacteriophage T4 lysozyme (T4L). T4L was chosen as a model system for this study because its structure (14), stability and activity (15, 16), and fluorescence properties (17) are all well characterized, and a variety of unnatural amino acids have been incorporated into T4L already by using unnatural amino acid mutagenesis (13, 18, 19). Herein we report the successful site-specific incorporation of the spin-labeled amino acid **1** (see Table 1 for structures), the fluorescent amino acid 7-azatryptophan (z^{7}Trp), **4**, and the photoaffinity label **6** at several external and internal sites in T4L by using unnatural amino acid mutagenesis.

MATERIALS AND METHODS

Mutagenesis. Restriction enzymes were purchased from New England Biolabs; T4 DNA ligase was from Promega; reagents and enzymes for DNA mutagenesis and sequencing were obtained from Amersham and United States Biochemical, respectively. Oligonucleotides were synthesized on an Applied Biosystems PCR Mate DNA synthesizer. Amber mutants were constructed by Eckstein mutagenesis (20) using an M13mp18 derivative containing a 650-bp *Bam*HI/*Hin*dIII fragment from plasmid pHSe54,97.TA, which encodes a cysteine-free T4L behind a twin *tac* promoter (21). The oligonucleotides 5′-AGC-TTT-ATC-TAA-TTC-CTA-TTT-AGC-AGC and 5′-TGT-TTG-ATT-ATA-CTA-TCT-ACT-TTT-AGC were used to generate the pT4L44am and pT4L138am mutants, respectively. Mutations were verified by dideoxynucleotide sequencing (22). Plasmids pT4L82am, pT4L129am, pT4L133am, and pT4l153am were provided by J. Ellman and D. Mendel (University of California, Berkeley).

Synthesis of Unnatural Amino Acids. *6-Nitroveratryloxycarbonyl(Nvoc)-L-2-amino-3-thiomethyl-1-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolin-3-yl)propanoate cyanomethyl ester.* The amino acid (0.67 mmol) was modified as described (23) to yield 150 mg (42%) of a yellow solid: R_f = 0.42 in 1:1 (vol/vol) ethyl acetate/hexane; MS, m/z 551.1 (MH$^+$); high-

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Abbreviations: T4L, bacteriophage T4 lysozyme; z^{7}Trp, 7-azatryptophan; Nvoc, 6-nitroveratryloxycarbonyl; Bpoc, biphenylisopropyloxycarbonyl; HR, high-resolution.

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resolution (HR) MS, calculated 551.181, found 551.180 (MH^+).

N-Nvoc-DL-2-amino-3-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolin-3-yl)propanoate cyanomethyl ester. The amino acid (0.24 mmol) was modified as described (23) to yield 70 mg (59%) of a yellow solid: $R_f = 0.38$ in 1:1 ethyl acetate/hexane; MS, m/z 505.3 (MH^+).

N-Nvoc-DL-7-azatryptophan cyanomethyl ester. The amino acid (2.0 mmol) was modified as described (23). The product was purified by preparative HPLC using a linear gradient of 10:90 to 90:10 (vol/vol) CH_3CN/50 mM NH_4OAc, pH 4.5, over 90 min to yield 0.35 g (36%) of a yellow solid: $R_f = 0.35$ in ethyl acetate; ^1H NMR (400 MHz, C^2HCl_3) δ 8.23 (d, J = 4, 1), 7.86 (d, J = 7, 1), 7.68 (s, 1), 7.05 (m, 1), 6.91 (s, 1), 5.75 (d, J = 8, 1), 5.56 (d, J = 15, 1), 5.46 (d, J = 15, 1), 4.80 (d, J = 16, 1), 4.65 (d, J = 16, 1), 3.92 (s, 3), 3.85 (s, 3), 3.63 (m, 1), 3.34 (d, J = 5, 2); MS, m/z 484.1 (MH^+); HR MS, calculated 484.147, found 484.147 (MH^+).

N^{α}-Biphenylisopropyloxycarbonyl(Bpoc)-N^{ε}-dansyl-L-lysine cyanomethyl ester. The amino acid (1.1 mmol) was modified as described (23, 24) to yield 0.19 g (27%) of a yellow solid: R_f = 0.40 in 1:1 ethyl acetate/hexane; ^1H NMR (400 MHz, dimethyl-d_6 sulfoxide) δ 8.44 (d, J = 9, 1), 8.29 (d, J = 9, 1), 8.08 (d, J = 6, 1), 7.88 (t, J = 6, 1), 7.61 (m, 7), 7.43 (m, 4), 7.34 (t, J = 7, 1), 7.23 (d, J = 7, 1), 4.95 (s, 2), 3.82 (dd, J = 14, 1), 2.81 (s, 6), 2.75 (dd, J = 12, 6, 2), 1.69 (s, 6), 1.47 (m, 2), 1.25 (m, 4); MS, m/z 656.3 (MH^+); HR MS, calculated 656.267, found 656.267 (MH^+).

5'-Phospho-2'-deoxycytidylyl(3'-5')-2'(3')-O-[N-Nvoc-L-2-amino-3-thiomethyl-1-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolin-3-yl)propanyl]adenosine. This compound was prepared as described (23): MS, m/z 1132.4 (MH^+); HR MS, calculated 1132.285, found 1132.285 (MH^+).

5'-Phospho-2'-deoxycytidylyl(3'-5')-2'(3')-O-[N-Nvoc-L-2-amino-3-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolin-3-yl)propanyl]-adenosine. This compound was prepared as described (23): MS, m/z 1108.3 (MNa^+), 1086.3 (MH^+); HR MS, calculated 1086.297, found 1086.301 (MH^+).

5'-Phospho-2'-deoxycytidylyl(3'-5')-2'(3')-O-[N-Nvoc-DL-7-azatryptophanyl]adenosine. This compound was prepared as described (23): MS, m/z 1063.6 (MH^+); HR MS, calculated 1063.235, found 1063.236 (MH^+).

5'-Phospho-2'-deoxycytidylyl(3'-5')-2'(3')-O-[N^{ε}-(dansyl)-lysyl]adenosine. The 5'-phospho-2'-deoxycytidylyl(3'-5')-2'(3')-*O*-[N^{α}-(Bpoc)-N^{ε}-(dansyl)lysyl]adenosine was prepared as with the *N*-Nvoc-protected amino acids. To remove the Bpoc protecting group, this product was dissolved in 1:1 double-distilled H_2O/CH_3CN (6 ml), and a solution of 0.5 M trifluoroacetic acid was added to the mixture until the pH reached 3. The mixture was heated for 2 hr in a 37°C water bath. The reaction was judged complete by analytical HPLC as for the protected aminoacyl pdCpA. The desired deprotected product was separated by preparative HPLC using a linear gradient of 10:90 to 90:10 CH_3CN/10 mM HOAc over 90 min. The pure product was stored as a solution at −80°C: MS, m/z 998.1 (MH^+); HR MS, calculated 998.263, found 998.263 (MH^+).

***In Vitro* Suppression and Synthesis of T4L.** All reagents and enzymes for *in vitro* synthesis reactions were purchased or prepared as reported, and the *in vitro* reactions were run as reported (9).

The S-30 extract containing the necessary enzymes for *in vitro* transcription and translation was prepared as described from *E. coli* strain D10 (*rna-10, relA1, spoT1, metB1*) with the following modifications (9). *E. coli* strain D10 was grown in a fermentor in 200 liters of LB (1% tryptone/0.5% yeast extract/1% NaCl in distilled H_2O) to an OD_{600} of 2 (late log phase, doubling time = 40 min). The cells were grown at the fermentation facility at the University of California, Berkeley. After the cells had been grown in LB, the cell pellet was resuspended in 4 ml of S-30 buffer per g of cell pellet prior to lysis. The 200-l preparation gave ≈1.8 l of the S-30 extract. The extract produces ≈30 μg of active T4L per ml, does not exhibit readthrough at low Mg^{2+} concentrations, and incorporates unnatural amino acids well.

The insoluble fractions of *in vitro* reactions were examined by dissolving the pellet from a 30-μl *in vitro* reaction in 20 μl of a Tris buffer [64 mM Tris·HCl, pH 6.8/10% (vol/vol) glycerol/2% (wt/vol) SDS/5% (vol/vol) 2-mercaptoethanol] at 95°C for 5 min and then visualizing the dissolved protein by SDS/PAGE and autoradiography. The suppression efficiency is defined as the ratio of the amount of T4L produced by suppressing a gene containing an amber codon *in vitro* relative to the amount produced from the wild-type gene *in vitro*. Readthrough is defined as the production of intact protein when aminoacyl-tRNA is replaced with unacylated tRNA in the *in vitro* reaction mixture.

Purification of T4L. T4L was synthesized *in vitro* (20–40 μg of wild-type protein per ml of *in vitro* reaction mixture) and purified to homogeneity in 5–10% overall yield (18). A clear supernatant was obtained by mixing the 5-ml *in vitro* reaction mixture with 100 μl of 5% (wt/vol) PEI·HCl, pH 7.5, on ice for 10 min, followed by centrifugation for 10 min at 4°C. The supernatant was dialyzed against 50 mM Tris·HCl, pH 7.5/1 mM EDTA (buffer 1) overnight at 4°C. The dialyzed supernatant was applied to tandem Millipore DEAE/CM-cellulose MemSep 1000 chromatography cartridges, which had been equilibrated in buffer 1, at a flow rate of 0.5 ml/min at room temperature. The columns were washed with 50 ml of buffer 1 at a flow rate of 1 ml/min at room temperature. The protein was eluted from the CM-cellulose cartridge by using a Pharmacia FPLC with a 30-ml linear gradient of 0 to 0.5 M NaCl in buffer 1 at a flow rate of 0.3 ml/min, and 1-ml fractions were collected. Fractions were analyzed for T4L by catalytic activity. Fractions containing activity were combined and dialyzed overnight against 50 mM NaOAc, pH 4.9/1 mM EDTA (buffer 2). The protein solution (2–5 ml) was concentrated to 1 ml by using an Amicon Centricon-10 concentrator (precoated with bovine serum albumin) and applied to a Mono S column (Pharmacia) equilibrated in buffer 2/0.15 M NaCl. The protein was eluted by using a Pharmacia SMART FPLC with a 2-ml linear gradient of 0.15 to 0.5 M NaCl in buffer 2 at a flow rate of 0.1 ml/min, and 100-μl fractions were collected. Fractions containing T4L were identified by the absorbance at 280 nm and by catalytic activity. Protein yields were determined by polyacrylamide gel electrophoresis with silver staining.

EPR and Fluorescence Spectra. For the EPR studies, the purified proteins were concentrated by using an Amicon Microcon-10 concentrator. The filter was precoated with 50 μl of purified wild-type T4L at 1 mg/ml in buffer 1, and the coated filter was then used to concentrate 200 μl of a solution of the mutant protein in the same buffer to approximately 20 μl. X-band EPR spectra were recorded on a Varian E-109 spectrometer modified for use with a loop gap resonator (25). This configuration is optimized for small volumes of aqueous samples.

The fluorescence spectra of the wild-type and Trp-138 → z^7Trp proteins were recorded from ≈0.1 μM protein solutions in buffer 1. The fluorescence spectra were recorded on a Perkin–Elmer model LS-5B Luminescence Spectrometer. The excitation wavelength was held constant at 288 nm, and the emission wavelength was scanned from 310 to 400 nm. All spectra are uncorrected, have the cell plus buffer background subtracted, and were recorded in triplicate.

RESULTS AND DISCUSSION

Incorporation of the Probes. The spin-labeled amino acids **1–3**, the fluorescent amino acids **4** and **5**, and the photoaf-

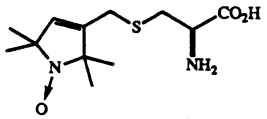
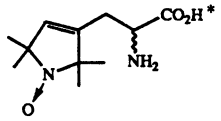
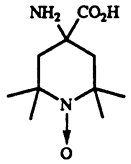
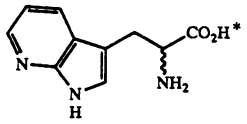
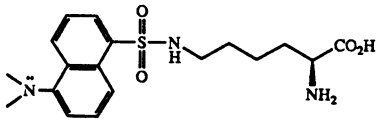
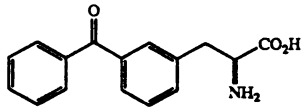
finity label **6** (see Table 1 for structures) were tested for incorporation at several surface and internal sites in T4L to demonstrate the generality of the method. The unnatural amino acids were substituted for two surface residues in T4L: Ser-44, which is in the middle of an α-helix, and Ala-82, which is at a break between two α-helices (14). Both of these sites have been shown to accommodate a number of natural and unnatural amino acids with little effect on T4L stability and activity (18, 19, 26, 27). In addition, four internal sites, Ala-129, Leu-133, Trp-138, and Phe-153, which surround the two largest hydrophobic pockets in T4L, were examined (14). Mutation of the residues surrounding these pockets has been shown to be accommodated by local structural rearrangements with little effect on T4L's overall structure or activity (16, 28).

The unnatural amino acids were designed to be similar in size to the natural amino acids to minimize structural perturbations to the protein and to have short linkers between the reporter group and the α-carbon to limit the conformational freedom of the probe. Spin labels were chosen that contain either the nitroxyl 1-oxyl-2,2,5,5-tetramethylpyrroline or 1-oxyl-2,2,6,6-tetramethylpiperidine (2). The fluorescent probe z^{7}Trp (**4**) was examined because it is known to be recognized by the *E. coli* protein biosynthetic machinery (29) and is reported to have absorption (288 nm) and emission (410 nm) maxima in water distinct from those of Trp (280 nm and 398 nm, respectively) (30, 31). In addition, Negerie *et al.* (30) report that z^{7}Trp and Trp can be excited selectively in water. The dansyl group (in **5**) was chosen because its absorption (340 nm) and emission (510 nm) maxima in water are quite distinct from those of the natural aromatic amino acids, it has a high quantum yield, and it has been used in combination with Trp in fluorescence-energy transfer experiments (3, 32). The benzophenone side chain (in **6**) was used because its excited-state triplet biradical shows selectivity for insertion into C—H bonds over reaction with water (33).

The spin labels **1–3**, the fluorescent probes **4** and **5**, and the photoaffinity label **6** were tested for incorporation at sites 44, 82, 129, 133, 138, and 153. Amber suppressor tRNA was charged with the unnatural amino acids **1–6**, and the aminoacylated suppressor tRNA was combined with mutant T4L genes containing amber codons at the desired positions in an *in vitro* transcription/translation system. Protein production levels were judged by catalytic activity and by SDS/PAGE and autoradiography of the soluble fraction (18). For each site tested, the amount of protein produced when the aminoacyl-tRNA was replaced by an unacylated tRNA, the read-through, was determined to be less than 1%.

The spin-labeled amino acid **1** could be incorporated at the external sites Ser-44 and Ala-82 with suppression efficiencies between 10% and 20% to produce catalytically active protein (Table 1, Fig. 1). When **1** was substituted at the internal sites Ala-129, Leu-133, Trp-138, and Phe-153, no protein of the correct molecular weight was found in the soluble fraction by SDS/PAGE and autoradiography, and no catalytic activity was detected. Protein of the correct molecular weight, how-

Table 1. Suppression efficiencies of the biophysical probes at different sites in T4L

Amino acid	No.	Ref.	Suppression efficiency* at site					
			44	82	129	133	138	153
	**1**	K.H., unpublished	+	+	−†	−	−	−†
	**2**	34	−	−	−	−	−	−
	**3**	35	−	−	−	−	−	−
	**4**		+++	+++	+++§	+++	+++	+++
	**5**		−	−	−	−	−	−
	**6**	33; J. Ellman, D.R.B., and P.G.S., unpublished	+++	+++	+++§	+++	+++§	+++

*+++, 30–80% suppression efficiency; +, 10–20%; −, less than 5%.
†These proteins were observed by SDS/PAGE in the insoluble fraction.
‡Previous results show that only the L isomer is recognized by the *E. coli* protein biosynthetic machinery (19).
§Proteins with these mutations are not catalytically active, possibly because the substituents interfere with correct folding or involve sites necessary for catalytic activity.

ever, was observed by SDS/PAGE and autoradiography in the insoluble fraction for mutants in both positions 129 and 153. These results suggest that substitution of **1** at internal sites may generate unstable protein or may impede correct folding of the protein, leading to aggregation or to degradation by proteases potentially present in the *E. coli* cell extract. Although they contain side chains similar to **1**, the spin labels **2** and **3** could not be incorporated at any of the sites tested as judged by enzymatic activity and by SDS/PAGE and autoradiography for both the soluble and insoluble fractions (Table 1, Fig. 1).

Both z^7Trp (**4**) and the benzophenone-derivatized amino acid **6** were inserted with between 30% and 80% efficiency at all sites tested (Table 1, Fig. 1). The Ala-129 → z^7Trp, Ala-129 → benzophenone, and Trp-138 → benzophenone mutants, however, were not catalytically active. These mutant proteins may be inactive because amino acids necessary for catalysis have been replaced, because the proteins are unstable, or because the proteins do not fold correctly. The dansyl amino acid **5** could not be incorporated at any of the sites tested as judged by enzymatic activity and by SDS/PAGE and autoradiography for both the soluble and insoluble fractions (Table 1, Fig. 1).

Factors Influencing Suppression Efficiency. The incorporation of the spin label **1** and the photoaffinity label **6**, both of which differ significantly from the natural amino acids, into T4L implies that the *E. coli* protein biosynthetic machinery can tolerate a wide range of amino acid side chains. The ability to incorporate amino acids with varied side chains in an *in vitro* protein synthesis system is consistent with earlier work using chemically modified aminoacyl-tRNAs (4, 6–8). A number of Lys analogues modified at the ε-amino group and Phe analogues have been incorporated when a wheat germ or rabbit reticulocyte extract was used to support *in vitro* translation.

There are, however, differences in the suppression efficiencies observed for particular amino acids by using the method of unnatural amino acid mutagenesis and by using the method of chemical modification of aminoacyl-tRNAs. For example, L-4′-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]phenylalanine was incorporated with less than 10% efficiency when unnatural amino acid mutagenesis was used (J. Ellman, D.R.B., and P.G.S., unpublished work), while the same amino acid acylated to *E. coli* $tRNA^{Phe}$ was estimated to be

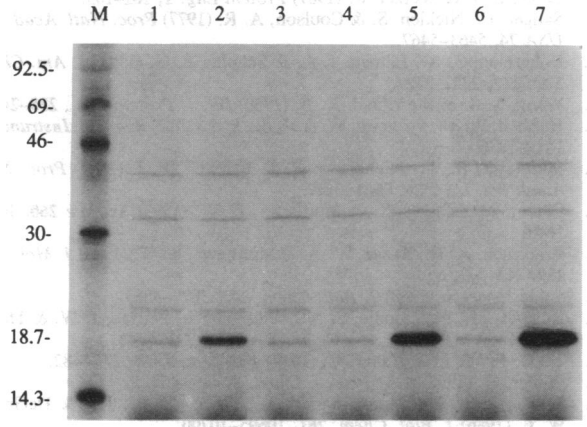


FIG. 1. Autoradiogram of *in vitro* suppression reaction products labeled with L-[^{35}S]methionine and containing pT4L44am and the following tRNAs: lane 1, full-length unacylated $tRNA_{CUA}$; lane 2, **1**-$tRNA_{CUA}$; lane 3, **2**-$tRNA_{CUA}$; lane 4, **3**-$tRNA_{CUA}$; lane 5, **4**-$tRNA_{CUA}$; lane 6, **5**-$tRNA_{CUA}$; and lane 7, **6**-$tRNA_{CUA}$. Lane M contains ^{14}C-methylated molecular weight ($\times 10^{-3}$) standards. Cleared supernatants (8 μl) from terminated 30-μl *in vitro* reaction mixtures were analyzed by SDS/15% polyacrylamide gel electrophoresis.

inserted into globin at 40% of its theoretical maximum by Baldini *et al.* (8). This result is not surprising, given the differences between the two systems. In unnatural amino acid mutagenesis, a chemically aminoacylated suppressor tRNA inserts an unnatural amino acid in response to a stop codon, UAG, using the protein biosynthetic machinery from an *E. coli* cell extract. In the method originated by Johnson, a chemically modified Lys-$tRNA^{Lys}$ or Phe-$tRNA^{Phe}$ is added to a wheat germ extract or a rabbit reticulocyte extract depleted of the natural aminoacyl-tRNA (4, 6–8). Simplistically, discrepancies between the two methods may reflect the way in which the suppression efficiencies or the levels of amino acid incorporation are calculated. Another possibility is that differences reflect dissimilarities in the specificity of amino acid or tRNA recognition between the prokaryotic *E. coli* protein biosynthetic machinery and the eukaryotic wheat germ and rabbit reticulocyte machinery (36). Moreover, the extent of amino acid incorporation may be lower when a suppressor tRNA, rather than one of the tRNAs aminoacylated by the 20 natural amino acids, is used, because the amber suppressor tRNA competes with a release factor which assists in the termination of protein synthesis for recognition of the amber codon. In fact, Miller and coworkers (37) report that the efficiency of natural amber suppressors in *E. coli* is improved *in vivo* when the gene for release factor 1 is deleted from the *E. coli* strain.

The variation in the suppression efficiencies of amino acids **1–6** at different sites was examined in relation to the context of the surrounding message. In independent studies, Miller and Albertini (38) and Bossi (39) have reported that for several natural amber suppressor tRNAs, the *in vivo* suppression efficiencies are higher when there is an A (or a G, to a lesser extent) on the 3′ side of the amber codon. The 5′ and 3′ contexts for sites 44, 82, 129, 133, 138, and 153 are listed in Table 2. For these sites, there is no obvious correlation between the adjacent codons and the suppression efficiencies. For example, although sites Ala-129 and Leu-133 both have an Ala, encoded by GCA and GCT, respectively, on their 3′ sides, protein containing spin label **1** at site 129 could be isolated, but not with the spin label at site 133. Rather than the codon context, the suppression efficiencies seen here are dominated by the amino acid identities. The benzophenone amino acid **6** was incorporated with >50% efficiency at all sites tested, while the spin-labeled amino acid **1** was incorporated with ≈10% efficiency at the identical sites. These results are corroborated by a survey of the suppression efficiencies of over 70 amino acids at more than 20 sites in different proteins using unnatural amino acid mutagenesis (40).

When the relationship between the amino acid identity and the suppression efficiency is analyzed, several correlations emerge. First, large hydrophobic amino acids such as z^7Trp (**4**), and the benzophenone derivative **6** were inserted with higher efficiency than small hydrophobic amino acids such as cyclopropylglycine or charged amino acids such as homoglutamate (40). This correlation is in agreement with reports that large, hydrophobic amino acids, such as Trp and Phe, bind more tightly to *E. coli* elongation factor Tu than do the other natural amino acids, such as Ala, Glu, and Lys (41). Second, amino acids significantly larger and longer than the

Table 2. Contexts of amino acid incorporation sites

Site	Codon	Codon to 5′ side	Codon to 3′ side
44	TCT (Ser)	AAA (Lys)	GAA (Glu)
82	GCT (Ala)	AAT (Asn)	AAA (Lys)
129	GCA (Ala)	GAA (Glu)	GCA (Ala)
133	TTA (Leu)	AAC (Asn)	GCT (Ala)
138	TGG (Trp)	AGA (Arg)	TAT (Tyr)
153	TTT (Phe)	ACG (Thr)	AGA (Arg)

natural amino acids, such as the benzophenone **6**, *O*-2-nitrobenzylserine, and aminoethylhomocysteine, were incorporated with high efficiency (40). Finally, the precise structure of an amino acid influences its suppression efficiency dramatically. Even though spin labels **1** and **2** both contain the 1-oxyl-2,2,5,5-tetramethylpyrroline group, only spin label **1**, which has a longer linker between the α-carbon and the pyrroline group, was incorporated. A caveat to these interpretations, however, is that the suppression efficiencies are determined based on the isolation of soluble stable protein and do not account for effects of the incorporated amino acid on the protein product.

Spectral Characterization. T4L mutants containing spin label **1** and z^{7}Trp (**4**) were purified to homogeneity and characterized to ensure that the probes were not being modified by the enzymes present in the S-30 extract and to determine how the spectral properties of the probes would be perturbed in the environment of a protein. T4L mutants with spin label **1** substituted for Ser-44 and z^{7}Trp substituted for Trp-138 were purified by elution from a CM-cellulose cartridge followed by elution from a Mono-S column in 5–10% overall yield. Close agreement in protein quantification by SDS/PAGE and catalytic activity suggests that substitution of spin label **1** at site 44 and of z^{7}Trp (**4**) at site 138 does not disturb the structure of T4L.

The Ser-44 → **1** mutant, when purified to homogeneity, gave a T4L mutant containing a nitroxide which could be detected by EPR (Fig. 2*A*). An X-band EPR spectrum of the Ser-44 → **1** mutant was obtained with less than 10 μl of an ≈1 μM solution of spin-labeled T4L in a loop-gap resonator (25). This amount of spin is at the detection limit for an immobilized nitroxide with broad resonance lines, and signal averaging (36 scans) was required to achieve a signal-to-noise ratio of about 2:1 for the most intense line. The resonance centered at $g = 2.006$, typical for the $M_I = 0$ line of a nitroxide in water, is clearly distinguished, but the $M_I = \pm 1$ lines are lost in the noise. The width of the $M_I = 0$ line (≈5 gauss) and the absence of equally intense resonances corresponding to $M_I = \pm 1$ indicate that the nitroxide is immobilized, with a correlation time > 4 ns, consistent with a protein-bound species.

Replacement of the Trp at site 138 with z^{7}Trp (**4**), leaving Trp-126 and Trp-158 in place, results in a red shift of ≈10 nm and broadening in T4L's emission maximum when excited at 288 nm (Fig. 2*B*). This result contrasts with reports in the literature that the spectra of indole and 7-azaindole can be resolved in an aqueous environment (30). The altered fluorescence properties are consistent, however, with reports that the three Trp residues in T4L interact photochemically with one another and with surrounding residues (17). These results suggest that the spectra of Trp and z^{7}Trp may be difficult to resolve when examined simultaneously in a protein environment rather than isolated in aqueous solution.

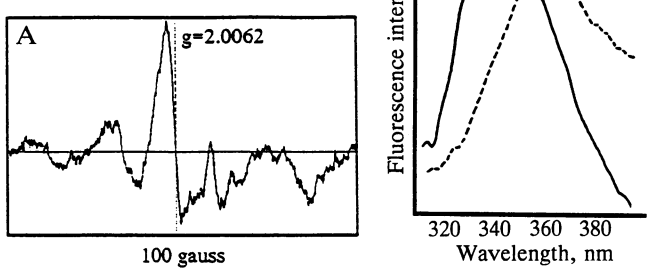


FIG. 2. (*A*) X-band EPR spectrum of the T4L mutant Ser-44 → spin label **1**. (*B*) Fluorescence emission spectra (uncorrected) of wild-type T4L (solid line) and of the T4L mutant Trp-138 → z^{7}Trp, **4** (broken line), excited at 288 nm.

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