couplings observed between the phosphorus and C-3 ($^3J_{C-P}$ = 3.7 Hz) and C-2 ($^2J_{C-P}$ = 9.5 Hz).

Incubation of CPEP with CPEP phosphonomutase[13] resulted in the formation of PPA which was identified by comparison with an authentic sample. The reaction was also followed by $^{31}$P NMR which showed appearance and increase of only one signal assignable to the phosphorus in phosphinopyruvate ($\delta_P$ 19.1) with the accompanied decrease of the signal due to CPEP.[14] Thus CPEP phosphonomutase is considered to catalyze an intramolecular rearrangement of CPEP to form the C-P bond of phosphinopyruvate (step 5b in Scheme I). Unlike the reaction catalyzed by PEP mutase, the concomitant decarboxylation probably drives the accumulation of the C-P compound. On the basis of these experimental results, the step 5 proved to consist of two reactions 5a (transesterification) and 5b (rearrangement and decarboxylation), and the blocked sites of NP71 and NP213 turned out to be (5a) and (5b) in Scheme I, respectively.

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(13) The reaction mixture (10 mL) containing 50 mM MES buffer (pH 6.5), 2 mM $MnCl_2$, 10 mM CPEP, and 10 units of CPEP phosphonomutase was incubated at 30 °C for 3 h. The product was purified by DEAE Sephadex A-25 and Sephadex G-10 column chromatographies.

(14) The reaction was carried out as described in ref 13, and the reaction mixture was analyzed after ultrafiltration by $^{31}$P NMR after 1, 2, and 3 h of incubation.

## Biosynthetic Site-Specific Incorporation of a Non-Natural Amino Acid into a Polypeptide

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Despite the enormous significance of site-specific mutagenesis in protein research, this technique suffers from the major limitation that substitutions are confined to the 20 primary amino acids normally present in proteins. As a result, the introduction of chemically unique residues into proteins is not possible with this approach. In spite of the development of strategies for circumventing this limitation,[1] a truly general method of site-specific protein modification would almost certainly require intervention during translation, in order to avoid the innate chemical selectivity problems associated with posttranslational modification. In this paper, a potential means of incorporating non-natural amino acids at specified sites during protein biosynthesis is described.

Biosynthetic incorporation of a non-natural amino acid has been achieved during normal translation with an appropriate "misacylated tRNA",[2] but that procedure results in the insertion of the amino acid at multiple sites—in competition with the cognate amino acid—because both the acylated tRNA and the wild-type tRNA compete for the same codon. The same would be true for all other codons except the three termination codons (UAG, UGA, and UAA), for which there are no corresponding tRNAs. Because these codons normally terminate translation, point mutations that insert any of them at an inappropriate site in a vital gene lead to nonfunctional products and cellular death.[3] In these instances, "read-through"—random insertion of an amino acid—cannot effectively compete with termination, and truncated protein is the major translated product. However, in some mutants, suppressor tRNAs have arisen that specifically recognize the misplaced termination codon (i.e., a *nonsense suppression site*) and thereby increase the amount of functional protein that is produced. This theme has evolved even further in an *Escherichia coli* strain that actually produces a UGA suppressor tRNA that incorporates a non-natural amino acid, selenocysteine, into formate dehydrogenase during translation.[4] Development of an analogous synthetic version of this theme could be accomplished by construction of a "chemically misacylated" nonsense suppressor tRNA employing methods developed by the Hecht group.[5] The addition of this acylated tRNA to a translation system containing a gene with a nonsense suppression site would result in the incorporation of the non-natural amino acid at the corresponding site in the protein. This strategy has recently been reported by the Schultz group, who employed a chemically misacylated yeast nonsense suppressor tRNA, prepared by anticodon loop replacement, to incorporate several phenylalanine analogues into $\beta$-lactamase.[6]

We have independently assessed a similar strategy to incorporate the non-natural amino acid iodotyrosine into a polypeptide. A relatively simple target, a 16-residue polypeptide, was initially chosen in order to quantitatively and unambiguously assess suppression efficiency, read-through, and site-specificity for this process. Accordingly, we prepared an acylated tRNA $^{125}$I-Tyr-tRNA$^{Gly}_{CUA}$-dCA[7] without base hypermodifications[8] to avoid the need for laborious isolation from a cellular source as previously performed[5,6] and with it have demonstrated high suppression efficiency with no detectable read-through, as well as unequivocal site-specificity of incorporation of the non-natural amino acid [$^{125}$I]-tyrosine. The construction of $^{125}$I-Tyr-tRNA$^{Gly}_{CUA}$-dCA was accomplished by (1) chemical synthesis of a 2′(3′)-O-acylated dinucleotide,[9] (2) run-off transcription of tRNA$^{Gly}_{CUA}$-C$_{OH}$,[10] and (3) enzymatic coupling of these fragments. Acylation of the protected dinucleotide with tyrosine[11] was achieved in high yield,

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(1) (a) Hilvert, D.; Hatanaka, Y.; Kaiser, E. T. *J. Am. Chem. Soc.* **1988**, *110*, 682-689. (b) Kaiser, E. T. *Ann. N. Y. Acad. Sci.* **1985**, *434*, 321-326. (c) Kaiser, E. T.; Lawrence, D. S. *Science* **1984**, *226*, 505-511. (d) Slama, J. T.; Radziejewski, C.; Oruganti, S.; Kaiser, E. T. *J. Am. Chem. Soc.* **1984**, *106*, 6778-6785.

(2) Baldini, G.; Martoglio, B.; Schachenmann, A.; Zugliani, C.; Brunner, J. *Biochemistry* **1988**, *27*, 7951-7959.

(3) Celis, J. E.; Smith, J. D. *Nonsense Mutations and tRNA Suppression*; Academic Press: New York, 1979.

(4) (a) Leinfelder, W.; Zehelein, E.; Mandrand-Berthelot, M.-A.; Bock, A. *Nature* **1988**, *331*, 723-725. (b) Söll, D. *Nature* **1988**, *331*, 662-661.

(5) This idea was apparently first suggested four years ago [(a) Shih, L. B.; Bayley, H. *Anal. Biochem.* **1985**, *144*, 132-141] based upon extensive work by the Hecht group [(b) Hecht, S. M.; Alford, B. L.; Kuroda, Y.; Kitano, S. *J. Biol. Chem.* **1978**, *253*, 4517-4520. (c) Heckler, T. G.; Zama, Y.; Naka, T.; Hecht, S. M. *J. Biol. Chem.* **1983**, *258*, 4492-4495. (d) Heckler, T. G.; Chang, L.-H.; Zama, Y.; Naka, T.; Chorghade, M. S.; Hecht, S. M. *Biochemistry* **1984**, *23*, 1468-1473. (e) Roesser, J. R.; Chorghade, M. S.; Hecht, S. M. *Biochemistry* **1986**, *25*, 6361-6365].

(6) Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Science* **1989**, *244*, 182-188.

(7) tRNA$^{Gly}_{CUA}$-C$_{OH}$: tRNA in vitro suppressor run-off transcript, based upon *E. coli* tRNA$^{Gly}_3$, which is missing the 3′-terminal cytidine and adenosine moieties; $^{125}$I-Tyr-tRNA$^{Gly}_{CUA}$-dCA: semisynthetic tRNA containing a deoxycytidine residue coupled to an adenosine moiety on the 3′-terminus and acylated with 3-[$^{125}$I]-tyrosine.

(8) tRNA hypermodification is an enzymatic process whereby specific nucleotides along the tRNA sequence are structurally modified posttranscriptionally.

(9) The synthesis of the acylated dinucleotide is based upon prior work developed by the Chládek group [(a) Hagen, M. D.; Scalfi-Happ, C.; Happ, E.; Chládek, S. *J. Org. Chem.* **1988**, *53*, 5040-5045. (b) Happ, E.; Scalfi-Happ, C.; Chládek, S. *J. Org. Chem.* **1987**, *52*, 5387-5391. (c) Scalfi-Happ, C.; Happ, E.; Ghag, S.; Chládek, S. *Biochemistry* **1987**, *26*, 4682-4688] with slight variations—to be reported in a later paper.

(10) The tRNA$^{Gly}_{CUA}$-C$_{OH}$ was prepared by simple run-off transcription from a synthetic linear DNA template featuring a double-stranded promoter region and a long 5′-overhang corresponding to the transcribed sequence [(a) Francklyn, C.; Schimmel, P. R. *Nature* **1989**, *337*, 478-481. (b) Milligan, J. F.; Groebe, D. R.; Witherell, G. W.; Uhlenbeck, O. C. *Nucleic Acids Res.* **1987**, *15*, 8783-8798].

0002-7863/89/1511-8013$01.50/0 

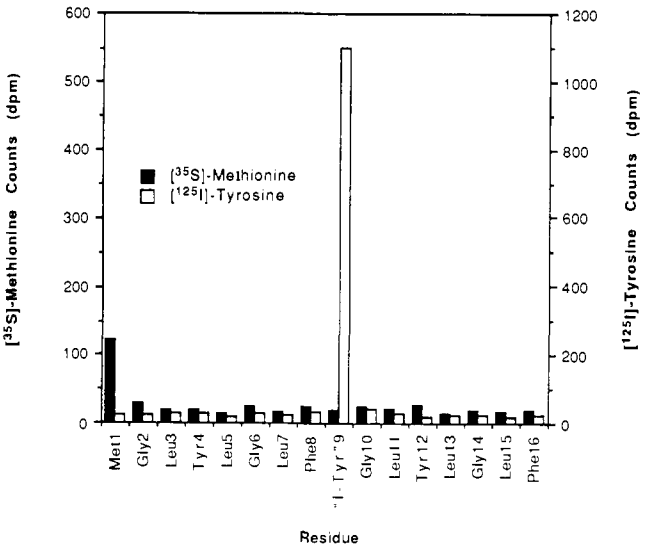


**Figure 1.** Amino acid sequence determination of the 16-residue polypeptide product from in vitro translation with rabbit reticulocyte lysate. Position 1 corresponds to the amino terminus (methionine), and position 9 corresponds to the suppression site (suppressed by $^{125}$I-Tyr-tRNA$^{Gly}_{CUA}$-dCA) of the polypeptide. All values are reported in dpm and have been corrected for incomplete PTH-derivatization and hydrolysis, in addition to cycle losses that occur during sequencing. Values are not corrected for background.

followed by deprotection[9b] and reverse-phase HPLC purification to give the fully deprotected acylated dinucleotide in 54% overall yield. The preparation of Tyr-tRNA$^{Gly}_{CUA}$-dCA was completed by T4 RNA ligase-mediated coupling of 20 molar equiv of the acylated dinucleotide with tRNA$^{Gly}_{CUA}$-C$_{OH}$.[2,5d] Radiolabeling of Tyr-tRNA$^{Gly}_{CUA}$-dCA with carrier-free Na[$^{125}$I] followed by chromatographic purification gave $^{125}$I-Tyr-tRNA$^{Gly}_{CUA}$-dCA.[12] Introduction of the radiolabel was conducted subsequent to T4 RNA ligation to minimize the number of steps that required the handling of radioactive material; however, other misacylated tRNAs have been prepared directly from the non-natural amino acid itself.[9]

The translation experiment[13] was conducted with this synthetic acylated tRNA and an appropriately designed mRNA containing a UAG termination codon at position 9, transcribed from a synthetic gene. Two polypeptides can be translated from this mRNA, either an 8-mer or a 16-mer (refer to Figure 1 abscissa), depending upon whether or not suppression occurs. Nonradiolabeled polypeptide standards of the 8- and 16-mer, synthesized by standard solid phase methods,[14] were added as carriers following termination of the translation reaction. Isolation was facilitated by the hydrophobic nature of both products and was accomplished quantitatively by extraction of the rabbit reticulocyte lysate with $CH_3CN/CHCl_3$ (1:1). After isolation, the extracts were fractionated by reverse-phase HPLC and radioisotope levels detected by scintillation counting. *The results of this experiment clearly show that the non-natural amino acid iodotyrosine[12] has been efficiently incorporated exclusively at the designated position.* Total suppression was calculated from the relative levels of [$^{35}$S]-methionine contained in the 8- and 16-mer polypeptide products (586 and 248 dpm, respectively) to give a value of 30% suppression efficiency. Furthermore, read-through is insignificant at this level of suppression, although small amounts of the 16-mer (3-4%) are produced by read-through in the absence of added $^{125}$I-Tyr-tRNA$^{Gly}_{CUA}$-dCA (controls, Table I).

Table I. Suppression Efficiency and Controls

| | decays per minute[a] (dpm) | | | |
|---|---|---|---|---|
| | 8-mer | | 16-mer | |
| experiment[b] | [$^{35}$S]-Met | [$^{125}$I]-Tyr | [$^{35}$S]-Met | [$^{125}$I]-Tyr |
| $^{125}$I-Tyr-tRNA$^{Gly}_{CUA}$-dCA | 586 | - | 248 | 2120 |
| control 1[c] | 836 | - | 26 | - |
| control 2[c] | 863 | - | 37 | - |
| control 3[c] | 815 | - | 23 | - |

[a] Counts per min have been converted into decays per min (dpm) and are corrected for background. Dashes (—) indicate no dpm above background levels. [b] In vitro rabbit reticulocyte lysate translation experiments were carried out as described in ref 13. Crude polypeptides were injected onto a Vydac C-4 column equilibrated in 0.1% TFA in $H_2O/CH_3CN$ (1:1). Gradient elution with 0.1% TFA in $CH_3CN$ (50-100% $CH_3CN$ in 30 min) resolved the 8- and 16-mer peaks eluting at 60-80% $CH_3CN$. [c] Controls were conducted in the absence of added $^{125}$I-Tyr-tRNA$^{Gly}_{CUA}$-dCA but were supplemented as follows: Control (1) buffer alone, control (2) 0.5 μg of nonacylated tRNA, and control (3) 0.5 μg of nonacylated tRNA plus 100 μCi carrier-free [$^{125}$I]-tyrosine.

Finally, in order to rigorously demonstrate the site-specificity of this process, the HPLC-purified 16-mer was sequenced by standard gas-phase analysis. Phenylthiohydantoin (PTH) derivatives were identified by reverse-phase HPLC followed by detection of both [$^{35}$S]-methionine and [$^{125}$I]-tyrosine through scintillation counting of the individual fractions (Figure 1). Only position 9, the expected site of suppression, contained $^{125}$I above background levels, unequivocally establishing that [$^{125}$I]-tyrosine is incorporated exclusively at that position. These studies are currently being expanded to include the incorporation of a variety of other amino acids, the introduction of several different residues into the same protein, and an in vivo translation system.

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(11) Hudson, D. *J. Org. Chem.* **1988**, *53*, 617-624.
(12) The reaction was carried out under conditions to minimize nucleotide substitution (Scherberg, N. H.; Seo, H.; Hynes, R. *J. Biol. Chem.* **1978**, *253*, 1773-1779).
(13) A typical reaction (10 μL) contained commercially available rabbit reticulocyte lysate (8 μL), L-[$^{35}$S]-methionine (1 μL, 100 μCi), mRNA (0.5 μg in 0.5 μL of $H_2O$), $^{125}$I-Tyr-tRNA$^{Gly}_{CUA}$-dCA (5 μg), and $H_2O$ (0.5 μL). The mixture was incubated for 1 h at 30 °C followed by addition of 1.0 M sodium hydroxide/hydrogen peroxide (0.5 mL) and warming to 37 °C for 10 min.
(14) Dryland, A.; Sheppard, R. C. *J. Chem. Soc., Perkin Trans. 1* **1986**, 125-137.

## Intermolecular Pinacol Cross Coupling of Electronically Similar Aldehydes. An Efficient and Stereoselective Synthesis of 1,2-Diols Employing a Practical Vanadium(II) Reagent

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The 1,2-diol unit is one of the most ubiquitous functional groups in nature, and consequently a wealth of methods leading to its synthesis have been developed. Foremost in this arsenal are the catalytic osmylation of olefins,[1] ring opening of epoxides,[2] and reduction or alkylation of α-hydroxy/alkoxy carbonyls.[3] Common

(1) (a) Wai, J. S. M.; Marko, I.; Svendsen, J. S.; Finn, M. G.; Jacobsen, E. N.; Sharpless, K. B. *J. Am. Chem. Soc.* **1989**, *111*, 1123. (b) Schroder, M. *Chem. Rev.* **1980**, *80*, 187.
(2) (a) Behrens, C. H.; Sharpless, K. B. *J. Org. Chem.* **1985**, *50*, 5696. (b) Behrens, C. H.; Ko, S. Y.; Sharpless, K. B.; Walker, F. J. *Ibid.* **1985**, *50*, 5687. (c) Caron, M.; Sharpless, K. B. *Ibid.* **1985**, *50*, 1557. (d) Gorzynski Smith, J. *Synthesis* **1984**, 629. (e) Behrens, C. H.; Sharpless, K. B. *Aldrich. Acta* **1983**, *16*, 67.

0002-7863/89/1511-8014$01.50/0 