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Enhanced D-Amino Acid Incorporation into Protein by Modified Ribosomes

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The use of purposefully misacylated tRNAs[1] in ribosomally mediated protein synthesizing systems has enabled the elaboration of proteins containing nonproteinogenic amino acids.[2] While most of these have involved simple alteration of the C^{α} side chain, there are also examples of more fundamental alterations of amino acid structure.[3] The latter hold great promise for the preparation of novel biomaterials and the detailed analysis of protein function, but are limited by the marginal ability of the ribosome to utilize the corresponding activated tRNAs. For example, D-amino acids are widely distributed in living organisms and their active utilization in many cell types has been demonstrated,[4] but all D-amino acid-containing peptides and proteins result from posttranslational modification[4a,5] or from nonribosomal synthesis.[6] Attempts to incorporate D-amino acids into proteins using chemically misacylated suppressor tRNAs in cell-free protein synthesizing systems have been unsuccessful.[7]

A novel strategy for enhanced incorporation of D-amino acids into protein might involve alteration of the peptidyltransferase center (PTC). Recent work has demonstrated that mutations in 23S rRNA in the region of the PTC (position 2447 and 2449) led to the formation of ribosomes having translation properties different from those of wild type.[8] We attempted to utilize analogous ribosome modifications to facilitate the incorporation of D-amino acids into protein.

By overrexpression of modified 23S rRNAs from multicopy plasmids, we prepared modified ribosomes having mutations in two regions (2447−2450 and 2457−2462) of *Escherichia coli* 23S rRNA;[9] these correspond to the PTC and helix 89, respectively.[10] The cells containing mutant ribosomes grew slowly; therefore, to increase the probability of selecting mutants we employed primers for PCR having random (but lacking wild-type nucleotide) sequences in the (2447−2450 or 2457−2462) regions of interest.[11] During mutagenesis and further selection in the presence of ampicillin and variable chloroamphenicol concentrations, two clones having mutations in the 2447−2450 region (PTC) and six with mutations in the 2457−2462 region (helix 89) were prepared and sequenced. A high level of homology in their sequences was observed (Table 1).[12]

Cell-free protein synthesizing systems (S-30) were prepared from cultures of six *E. coli* mutants having different mutant ribosomes.[13] These S-30 preparations were analyzed for their abilities to incorporate D-Met and D-Phe into proteins in vitro. An S-30 preparation containing wild-type ribosomes was used as a control (Table 1 and Figure 1). The D-methionyl and D-phenylalanyl-$tRNA_{CUA}$s were prepared as described.[14]

The incorporation of D-amino acids into protein was studied initially in terms of the extent of the UAG codon suppression in the *E. coli* DHFR and *Photinus pyralis* firefly luciferase mRNAs.[15] As illustrated in Figure 1 for the synthesis of DHFR from a mRNA containing a UAG codon at position 22, the level of protein synthesis in the presence of L-methionyl-$tRNA_{CUA}$ and L-phenylalanyl-$tRNA_{CUA}$ was about 50% of that of protein synthesis from unmodified mRNA. Using S-30 preparations having mutant ribosomes resulted in higher levels of suppression in the presence of both D-methionine (23%) and D-phenylalanine (12%). Experiments involving DHFR mRNAs containing UAG codons in two other positions (10 and 54) and firefly luciferase mRNAs containing UAG codons at positions 247 or 250 reinforced the results shown in Figure 1 (not shown); suppression efficiencies obtained with D-methionyl-$tRNA_{CUA}$ and D-phenylalanyl-$tRNA_{CUA}$ were typically 35−45% of those obtained using the respective L-aminoacyl-$tRNA_{CUA}$s.[16]

Also determined were the specific activities of the proteins containing D-methionine and D-phenylalanine. Two types of positions were chosen for modification. The first were sites known to be situated near the protein active site and to participate in protein function. These included Trp22 and Leu54 for DHFR[17] and Phe247 for firefly luciferase.[18] The other positions involved amino acids believed not to be important for protein function, namely Val10 in DHFR and Phe250 in luciferase. As shown in Figure 2, the introduction of D-methionine into position 54 of DHFR greatly

Table 1. Characteristics of Cell-Free Protein Synthesizing Systems Prepared with Modified Ribosomes (Mutant Sequences in Boldface Type).

sequence in regions		mutant	suppression efficiency[a],%	
2447–2451	2457–2462	ribosome[13], %	D-Met	D-Phe
		wild type		
GAUAA	UGAUAC	no	5.3	2.8
		mutant		
UUGUA	UGAUAC	36	20.9	8.9
UGGCA	UGAUAC	43	22.9	11.1
GAUAA	**GCGGAU**	52	17.5	8.9
GAUAA	**CUGGAG**	55	16.9	11.5
GAUAA	**GCUGAU**	63	19.7	8.6
GAUAA	**GUGGAG**	40	11.1	4.4

[a] Determined as percent of full-length DHFR, formed by readthrough of a UAG codon at position 22, relative to the amount of full-length DHFR produced using wild-type mRNA. For wild-type mRNA, all of the S-30 preparations exhibited comparable levels of DHFR synthesis. The standard deviation in replicate experiments was ≤2.0.

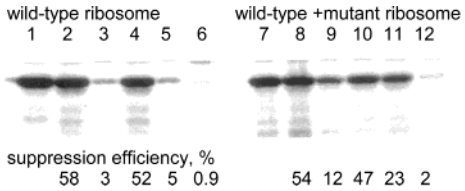

Figure 1. In vitro DHFR synthesis programmed by wild-type and mutant (TAG codon in position 22, corresponding to Trp 22) genes by the use of cell-free protein synthesizing systems containing ribosomes with wild-type or modified 23S rRNA (sequence UGGCA instead of GAUAA in the region 2447−2451). Lanes 1 and 7, synthesis from wild-type mRNA; lanes 2 and 8, L-phenylalanyl- $tRNA_{CUA}$; lanes 3 and 9, D-phenylalanyl-$tRNA_{CUA}$; lanes 4 and 10, L-methionyl -tRNA; lanes 5 and 11, D-methionyl -$tRNA_{CUA}$; lanes 6 and 12, nonacylated $tRNA_{CUA}$. Suppression efficiency was defined as percent of full-length DHFR, formed by readthrough of a UAG codon, relative to amount of full-length DHFR produced from wild-type mRNA.

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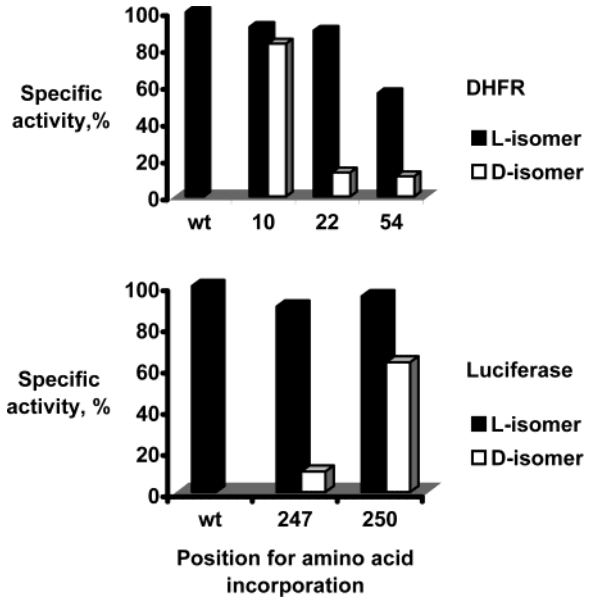

Figure 2. Comparison of specific activities of *E. coli* DHFR and *P. pyralis* firefly luciferase, synthesized from modified genes. L- and D-methionyl-$tRNA_{CUA}$'s were used for suppression of UAG codons in the positions corresponding to Val10 and Leu 54 of DHFR; L-and D-phenylalanyl $tRNA_{CUA}$'s were used for suppression of the UAG codons in positions corresponding to Trp22 of DHFR, and Phe247 and Phe250 of luciferase.

diminished the specific activity of DHFR, as did the inclusion of D-phenylalanine in position 22 of DHFR or 247 of luciferase. In comparison, DHFR containing D-methionine in position 10 functioned essentially as well as DHFR containing L-methionine (or wild-type valine) at that position. A similar result was obtained for the luciferases containing L- and D-phenylalanine at position 250.

Thus, we have demonstrated that modified ribosomes can facilitate the incorporation of D-amino acids into protein. The data are consistent with the interpretation that mutations in 23S rRNA in the region of the PTC and helix 89 lead to conformational changes in the ribosome that alter its behavior in protein synthesis. In the above cases, the putative alterations in ribosome structure may diminish the normal mechanisms that discriminate against D-aminoacyl-$tRNA_{CUA}$'s in the ribosomal A-site. More generally, it seems likely that analogous changes in key regions of 23S rRNA may permit the elaboration in the in vitro systems of proteins containing a much broader variety of amino acid analogues.

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(9) Sawano, A.; Miyawaki, A. *Nucleic Acids Res.* **2000**, *28*, e78. Plasmid pUCrrnB, used in the mutagenesis procedure, was constructed by incorporation of the rrnB operon, excised from plasmid pNot by *Kpn*I and *Bam*HI restriction endonucleases, and introduced into high-copy vector pUC18 under the control of an IPTG-induced Lac promoter.
(10) O'Connor, M.; Dahlberg, A. E. *J. Mol. Biol.* **1995**, *254*, 838. The latter region plays an important role in translation.
(11) The modified plasmids had the rrnB gene expressing β-lactamase, thus conferring resistance to ampicillin, and were expected to have variable resistance to chloramphenicol.[8a]
(12) All mutants in the region 2457−2462 had the substitution U2460G. This has been reported to increase misreading but not to lead to a lethal phenotype.[10] Three of six mutants had the same sequence, CUGGAG, instead of UGAUAC (wild-type). While the 23S rRNA sequences in Table 1 were inferred from DNA sequencing, the presence of the UUGUA sequence at positions 2447−2451 of the first mutant in the table was verified by hybridization of a radiolabeled DNA probe to the isolated 23S rRNA.
(13) S-30 systems were prepared essentially as described in J. M. Pratt, *Transcription and Translation: A Practical Approach*; IRL Press: Oxford, 1984; pp 179−209. The S-30 preparations of individual colonies were scored for their abilities to effect the incorporation of D-phenylalanine and D-methionine into DHFR by suppression of a UAG codon at position 22 with D-phenylalanyl-$tRNA_{CUA}$. The initial rrnB gene used in this work contained one point mutation in the 23S rRNA gene (A1061T), which confers thiostrepton resistance (Spahn, C. M. T.; Remme, J.; Schafer, M. A.; Nierhaus, K. H. *J. Biol. Chem.* **1996**, *271*, 32849). Therefore, the levels of plasmid-born ribosome in all S-30 preparations were estimated from the level of protein synthesis in the presence of this antibiotic and verified by the primer extension method (Table 1); they varied from 36 to 63%. The translational fidelity of all S-30 preparations was estimated by the relative enzyme activity of *E. coli* DHFR synthesized in the presence of thiostrepton, which was ∼80−85% of DHFR prepared using wild-type ribosomes.
(14) Lodder, M.; Golovine, S.; Laikhter, A. L.; Karginov, V. A.; Hecht, S. M. *J. Org. Chem.* **1998**, *63*, 794. D-Methionine and D-phenylalanine were treated with L-amino acid oxidase prior to attachment to the suppressor tRNA to destroy any residual L-amino acid.
(15) In vitro translation was carried out in the presence of rifampicin (10 mg/mL) to limit mRNA synthesis to those genes that were under the control of a T7 promoter. S-30 systems prepared from cells lacking plasmid-born ribosomes mediated only slight protein synthesis in the presence of D-methionyl-$tRNA_{CUA}$ and, especially, D-phenylalanyl-$tRNA_{CUA}$.
(16) That suppression of the UAG codons in the mRNAs actually had involved transfer of the aminoacyl moieties from the activated suppressor tRNAs was investigated using D- and L-methionyl-$tRNA_{CUA}$'s in a protein synthesizing system programmed with DHFR mRNA containing a UAG codon at position 42. The derived proteins were subjected to CNBr mapping, which verified the incorporation of D-methionine.
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