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Incorporation of β-amino acids into dihydrofolate reductase by ribosomes having modifications in the peptidyltransferase center

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ABSTRACT

Ribosomes containing modifications in three regions of 23S rRNA, all of which are in proximity to the ribosomal peptidyltransferase center (PTC), were utilized previously as a source of S-30 preparations for in vitro protein biosynthesis experiments. When utilized in the presence of mRNAs containing UAG codons at predetermined positions + β-alanyl–tRNA$_{CUA}$, the modified ribosomes produced enhanced levels of full length proteins via UAG codon suppression. In the present study, these earlier results have been extended by the use of substituted β-amino acids, and direct evidence for β-amino acid incorporation is provided. Presently, five of the clones having modified ribosomes are used in experiments employing four substituted β-amino acids, including α-methyl-β-alanine, β,β-dimethyl-β-alanine, β-phenylalanine, and β-(*p*-bromophenyl)alanine. The β-amino acids were incorporated into three different positions (10, 18 and 49) of *Escherichia coli* dihydrofolate reductase (DHFR) and their efficiencies of suppression of the UAG codons were compared with those of β-alanine and representative α-L-amino acids. The isolated proteins containing the modified β-amino acids were subjected to proteolytic digestion, and the derived fragments were characterized by mass spectrometry, establishing that the β-amino acids had been incorporated into DHFR, and that they were present exclusively in the anticipated peptide fragments. DHFR contains glutamic acid in position 17, and it has been shown previously that Glu-C endoproteinase can hydrolyze DHFR between amino acids residues 17 and 18. The incorporation of β,β-dimethyl-β-alanine into position 18 of DHFR prevented this cleavage, providing further evidence for the position of incorporation of the β-amino acid.

1. Introduction

The side chains of individual amino acid residues play important roles in the formation and stability of secondary structures in peptides and proteins. For example, some amino acids, such as leucine and alanine, have enhanced propensities for stabilizing α-helices.[1–3] It has been demonstrated that oligopeptides comprised of β-amino acids can also have significant helical propensities, some of which are not dramatically different in overall structure than peptides formed from α-amino acids.[4–7] Additionally, peptide analogues containing β-amino acids often show enhanced stability to proteolytic digestion, making them excellent candidates for use in peptidomimetic compounds.[8–11] Thus, β-amino acid incorporation into peptides and proteins is of significant interest due to their ability to create new biopolymers with unusual structural features. Because virtually all peptide analogues incorporating β-amino acids are currently prepared by chemical synthesis, the size of the modified polypeptides accessible for study is limited. The largest β-mimetic, synthesized by Gellman and co-workers, was only 17 amino acids long.[10] Recombinant DNA techniques, in combination with in vitro translation, could in principle enable the synthesis of larger polypeptides and proteins incorporating β-amino acids, but this approach is presently limited by the strict bias of the ribosome,[12,13] which efficiently accepts only α-L-amino acids for protein synthesis.

Our efforts to modify the architecture of the PTC have shown that it is possible to prepare ribosomal variants with greater adaptability in their acceptance of α-D[14,15] and β-L-amino acids.[16] In a recent study, eight variants of modified ribosomes, selected for resistance to erythromycin, were used to prepare a library containing mutations in two regions of the PTC. Screening of this library for clones sensitive to β-puromycin lead to the identification of ribosomal variants putatively capable of recognizing β-amino acids, while retaining the ability to synthesize wild-type *Escherichia coli* dihydrofolate reductase (DHFR) with good fidelity.[16] It was also shown using S-30 preparations from two ribosomal clones that suppression of UAG codons in DHFR mRNAs resulted in enhanced full size protein synthesis in the presence of β-alanyl–tRNA$_{CUA}$.[16] However, this preliminary result was obtained

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only using β-alanine and the incorporation of β-alanine was not verified by any direct method.

In the present study of modified ribosomes selected using β-puromycin, five modified ribosomes have been employed to characterize protein synthesis with four β^2 and β^3-substituted β-alanine derivatives, all of which have been incorporated into DHFR (Fig. 1). Three different positions in DHFR were utilized for incorporation of the modified amino acids with S-30 preparations from the five ribosomal clones, demonstrating unique patterns of β-amino acid incorporation by individual S-30 preparations.

Selected DHFRs were analyzed by mass spectrometry following trypsin digestion to verify the incorporation of the modified amino acids solely into the intended positions. It was also shown that incorporation of a β-alanine derivative into position 18 of DHFR blocked proteolytic digestion at the C-terminus of Glu-17 by Glu-C endoproteinase. It is anticipated that the ability to incorporate β-amino acids into specific positions in proteins may facilitate protein folding and stability through the intrinsic conformational biases associated with such amino acids, and may also confer stability against proteolysis at specific amino acid residues.

2. Results and discussion

2.1. Preparation of suppressor $tRNA_{CUA}$s activated with β-amino acids

Five β-amino acids were chosen for study (Fig. 1). These included β-alanine (**1**) and four C-substituted β-alanine derivatives. The latter included α-methyl-β-alanine (**2**), β,β-dimethyl-β-alanine (**3**), β-phenylalanine (**4**) and *β*-(*p*-bromophenyl)alanine (**5**). This permitted an initial assessment of the ability of the several ribosomal clones to tolerate β-alanine derivatives substituted on the α and β carbon atoms, and having either aliphatic or aromatic substituents. β-Alanine derivative **2** was purposefully prepared in racemic form, enabling the incorporation of either enantiomer to be detected.

Activation of suppressor $tRNA_{CUA}$ with each of the five β-amino acids was carried out as described previously for β-alanyl–$tRNA_{CUA}$.[16] As illustrated in Scheme 1 for β-(*p*-bromophenyl)alanyl–$tRNA_{CUA}$, N-protection of the commercially available amino acid with a pentenoyl protecting group was achieved by treatment with the N-hydroxysuccinimide derivative of pentenoic acid.[17,18] The N-protected amino acid was then activated as the cyanomethyl ester **6**;[19] the overall yield for the two steps was 38%. Amino acid derivative **6** was then employed for the aminoacylation of pdCpA (**7**),[20] which involved sonication in DMF solution in the presence of triethylamine, affording *N*-pentenoyl-β-*S*-(*p*-bromophenyl)alanyl-pdCpA (**8**) in quantitative yield.

Activated pdCpA derivative **8** was ligated to an abbreviated suppressor $tRNA_{CUA}$ transcript using T4 RNA ligase as described previously, affording *N*-pentenoyl-β-(*p*-bromophenyl)alanyl–$tRNA_{CUA}$ (**9**).[16] The N^{β} protecting group was removed by treatment with aqueous iodine immediately prior to use in protein synthesis.[17,18]

Also prepared analogously were activated pdCpA derivatives *N*-pentenoyl-3-amino-(*R*,*S*)-2-methylpropionyl-pdCpA (**8a**), *N*-pentenoyl-3-amino-3,3-dimethylpropionyl-pdCpA (**8b**) and *N*-pentenoyl-β-*S*-phenylalanyl-pdCpA (**8c**).

2.2. In vitro translation using β-amino acids with wild-type ribosomes

It was anticipated that wild-type *E. coli* ribosomes would be ineffective in incorporating β-amino acids into proteins. Nonetheless, it seemed important to verify this experimentally before studying the modified ribosomes. Accordingly, all five β-aminoacyl–$tRNA_{CUA}$s were compared with α-phenylalanyl–$tRNA_{CUA}$ and α-threonyl–$tRNA_{CUA}$ for their ability to suppress a UAG codon at position 49 of DHFR mRNA using an S-30 preparation derived from

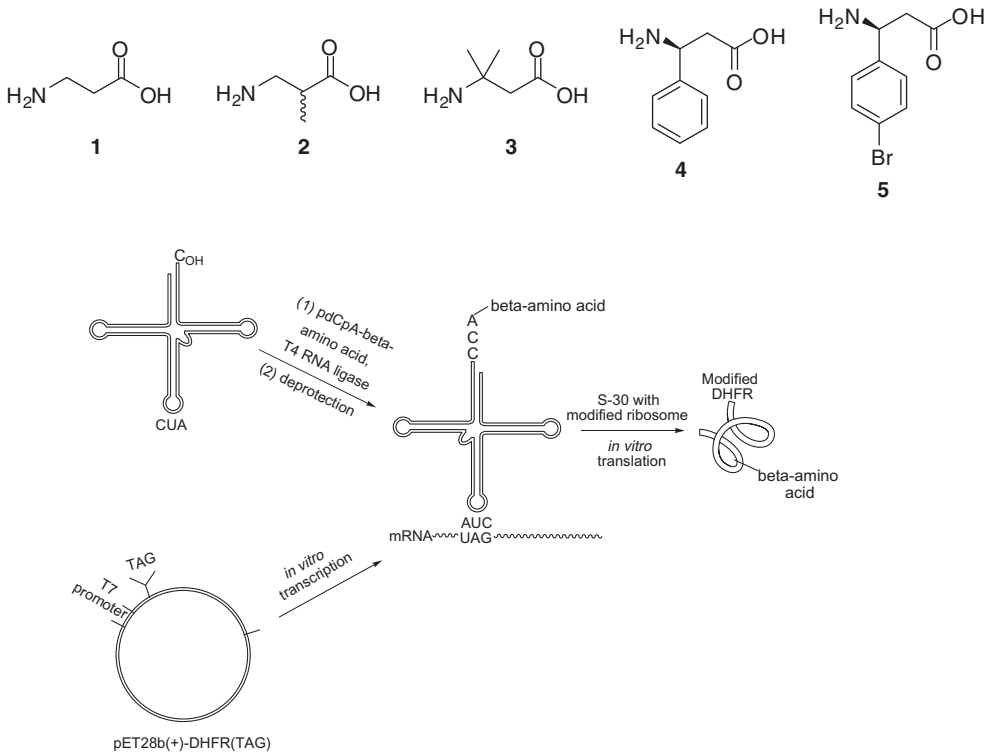
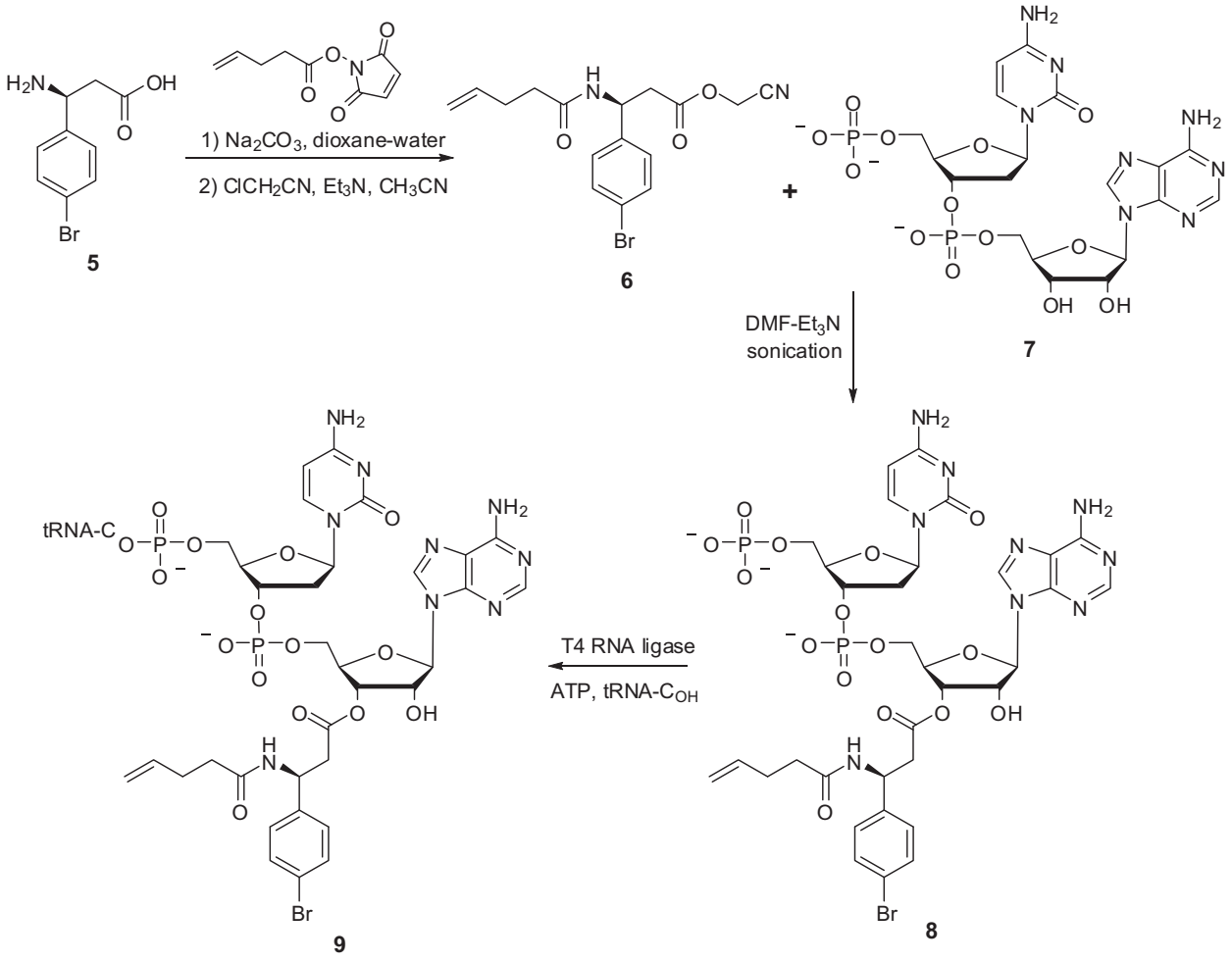


Figure 1. Structures of β-amino acids studied, and the strategy employed for their incorporation into DHFR using modified ribosomes.



Scheme 1. Synthesis of β-(*p*-bromophenyl)alanyl–$tRNA_{CUA}$.

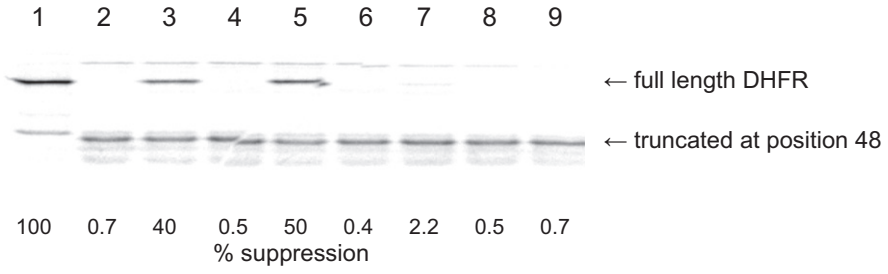


Figure 2. Translation of DHFR from wild-type (lane 1) and modified (lanes 2–9) (TAG codon in DHFR position corresponding to Ser49) genes in the presence of different suppressor $tRNA_{CUA}$s. Lane 2, nonacylated $tRNA_{CUA}$; lane 3, $tRNA_{CUA}$ activated with phenylalanine; lane 4, $tRNA_{CUA}$ activated with β-phenylalanine (**4**); lane 5, $tRNA_{CUA}$ activated with threonine; lane 6, $tRNA_{CUA}$ activated with β-(*p*-bromophenyl)alanine (**5**); lane 7, $tRNA_{CUA}$ activated with β,β-dimethyl-β-alanine (**3**); lane 8, $tRNA_{CUA}$ activated with α-methyl-β-alanine (**2**); lane 9, $tRNA_{CUA}$ activated with β-alanine (**1**). The suppression efficiency relative to wild type is shown below each lane.

wild-type ribosomes (Fig. 2). The suppression efficiency, expressed as a percentage of full length DHFR synthesized from the wild-type gene, was 40% for α-phenylalanyl–$tRNA_{CUA}$, 50% for α-threonyl–$tRNA_{CUA}$ and <2% above background for all of the β-amino acids, that is, much less than that of threonine and phenylalanine. Essentially the same result was obtained using DHFR mRNA containing a UAG codon at position 18 (Supplementary data, Fig. 1). These data demonstrated the inability of the wild-type ribosomes to incorporate these β-amino acids into protein with reasonable efficiency.

2.3. In vitro translation using β-amino acids with modified ribosomes

Five S-30 systems were prepared using different modified ribosome clones[16] (Table 1) after β-puromycin selection and were used for in vitro translation experiments. Two types of modified DHFR constructs, having a TAG codon in position Val10 (pETDH10 plasmid) or Asn18 (pETDH18 plasmid) were used initially for the attempted incorporation of the β-amino acids from their respective activated suppressor $tRNA_{CUA}$s. Because the efficiency of (unmodi-

Table 1
23S rRNA sequence modifications in PTC for clones used for S-30 preparations

Clone	Sequence in 23S rRNA of modified ribosome	
	region 1	region 2
0403x2	2057AGCGUGA2063	2502UUACCG2507
0403x4	2057AGCGUGA2063	2502AGCCAG2507
040321	2057AGCGUGA2063	2502AGAUAA2507
040329	2057AGCGUGA2063	2502UGGCAG2507
040217	2057AGCGUGA2063	2496AUAGUU2501
Wild type	2057GAAAGAC2063	2496CACCUC2501 2502GAUGUC2507

fied) DHFR synthesis from wild-type and each modified ribosomal preparation was different (Supplementary data, Table 1), the suppression efficiencies were expressed relative to the suppression obtained using α-threonyl–$tRNA_{CUA}$ (Table 2) or α-phenylalanyl–$tRNA_{CUA}$ (Table 3). DHFR synthesis obtained in the presence of non-acylated-$tRNA_{CUA}$ was used as the control for non-specific incorporation at the UAG codon. Quantification of DHFR synthesis was achieved by the use of a phosphorimager, which monitored the incorporation of ^{35}S-methionine.

As shown in Table 2 for incorporation into position 10 of DHFR, all five tested S-30 preparations gave only low levels (0.5–2.2%) of non-specific readthrough of the UAG codon in the absence of any activated suppressor tRNA. In comparison, UAG codon suppression in the presence of β-aminoacyl–$tRNA_{CUA}$s bearing β-amino acids **1**, **2** and **3** afforded β-amino acid incorporation in yields up to ~11%. Figure 2 (Supplementary data) illustrates the formation of full length DHFR using an S-30 system prepared from clone 040329 in the presence of β-amino acids **1**, **2** and **3**. Only slight variations in suppression efficiency (~2-fold) were observed for the individual β-amino acids using the five S-30 preparations having the modified ribosomes. The best DHFR yields were obtained using the S-30 preparations from clones 040329 and 040217.

Next, the incorporation of aromatic β-amino acids **4** and **5** into position 18 of DHFR was studied. β-Amino acid **2** was also included to facilitate a comparison with the results in Table 2. The protocol for the translation reactions was the same as in previous experiments and the suppression efficiency was expressed relative to that obtained using α-phenylalanyl–$tRNA_{CUA}$ (Table 3). β-Amino acid **2** gave comparable relative suppression efficiencies when using S-30 preparations from clones 0403x4 and 040329 (6.0% in both cases). However, minimal suppression was observed for the other S-30s preparations, in spite of the fact that **2** included both stereoisomers of the amino acid. S-30 preparations from clones 0403x4 and 040329 also afforded the best yield of DHFR for both β-amino acids with aromatic side chains **4** (13–18%) and **5** (13–15%). These clones have the same sequence in the first mutated region (2057AGCGUGA2063 vs 2057GAAAGAC2063 for wild-type ribosomes) and a high level of homology in the second mutated region (2502A**GC**U**CAG**2507 and 2502U**GG**U**CAG**2507), respectively. Interesting results were observed for S-30 system prepared from clone 040217, which has the same sequence as 0403x4 and 040329 in the first randomized region (2057–2063) of 23S rRNA, but a totally different second mutated region (2496–2501 vs 2502–2507) (Table 1). No significant difference was observed between the efficiency of incorporation of β-amino acids **1**, **3**, **4** or **5**. This S-30 preparation was not tested with β-amino acid **2** in the case of plasmid pETDH10, but in the case of pETDH18 essentially no full length DHFR synthesis was observed. Clones 0403x2 and 040321 have little homology with 0403x4 and 040329 in region 2 of the 23S rRNA (Table 1), and they did not demonstrate reasonable full length DHFR synthesis for β-amino acid **2** (1.5–3.8%) but for the other four β-amino acids the suppression efficiencies were moderate (5.3–7.9%).

Table 2
Incorporation of β-alanine analogues **1**, **2** and **3** into *E. coli* DHFR (position 10) by the use of S-30 systems having different modifications in the PTC

Amino acids	Suppression efficiency in different S-30 systems, having modified ribosomes (%)				
	040329	0403x2	0403x4	040321	040217
Thr	100	100	100	100	100
—	0.5[a] ± 0.3	2.1 ± 1.4	1.4 ± 0.7	0.9 ± 0.2	2.2 ± 1.0
1	8.2 ± 3.0	6.8 ± 3.2	8.1 ± 3.4	5.4 ± 1.3	9.9 ± 3.9
2	8.8 ± 1.0	3.8 ± 1.0	5.4 ± 1.9	Not tested	Not tested
3	11[b]	7.9 ± 1.1	7.1 ± 3.6	5.6 ± 3.0	10.9 ± 4.4

[a] Each number represents the average of three independent experiments ± SD.
[b] Tested in a single experiment (Supplementary data Fig. 2).

Table 3
Incorporation of β-alanine analogues **2**, **4** and **5** into *E. coli* DHFR (position 18) by the use of S-30 systems having different modifications in the PTC

Amino acids	Suppression efficiency in different S-30 systems, having modified ribosomes (%)				
	040329	0403x2	0403x4	040321	040217
Phe	100	100	100	100	100
—	1.5[a] ± 0.7	1.2 ± 0.4	2.4 ± 1.0	0.8 ± 0.4	1.0 ± 0.4
2	6.0 ± 0.5	1.5 ± 1.0	6.0 ± 2.2	1.3 ± 0.7	0.73 ± 0.2
4	18.4 ± 2.7	5.8 ± 1.3	13.2 ± 1.1	6.0 ± 1.8	7.8 ± 0.1
5	13.5 ± 1.5	5.3 ± 0.9	15.5 ± 2.0	7.6 ± 2.7	9.2 ± 0.4

[a] Each number represents the average of three independent experiments ± SD.

2.4. Suppression of UAG codon in three different positions of the DHFR gene by β-(*p*-bromophenyl)alanyl–$tRNA_{CUA}$

To study the ability of a single β-aminoacyl–$tRNA_{CUA}$ to suppress different positions on an mRNA, three plasmids (pETDH10, pETDH18 and pETDH49) have been tested comparatively for in vitro translation using an S-30 system prepared from clone 040217 and β-(*p*-bromophenyl)alanyl–$tRNA_{CUA}$ prepared from β-amino acid **5** (Fig. 3). β-Amino acid **5** was chosen because it was found to incorporate well into DHFR position 18 (Table 3), and because the bromine atom could confer an isotopic pattern in subsequent mass spectrometric analysis (Fig. 4). As shown in the Figure, relative to the suppression obtained using α-phenylalanyl–$tRNA_{CUA}$, β-(*p*-bromophenyl)alanine (**5**) was incorporated into positions 10, 18 and 49 in yields of 11%, 7.4% and 6.3%, respectively. Thus incorporation could be achieved in reasonable yields in a few different positions of DHFR.

2.5. Characterization of β-amino acids in DHFRs by MALDI analysis of peptides resulting from 'in-gel' trypsin digestion

In order to provide direct evidence for the incorporation of β-amino acids into DHFR, we prepared two modified DHFRs on a larger scale. The first employed plasmid pETDH49 and β-(*p*-bromo-

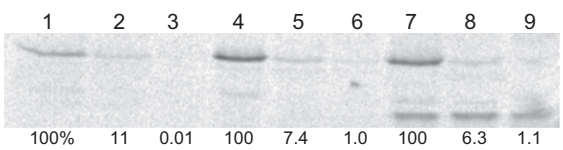


Figure 3. Relative suppression efficiency of β-(*p*-bromophenyl)alanyl–$tRNA_{CUA}$ at three different positions (10, 18 and 49) in DHFR, using an S-30 system prepared from clone 040217. Lanes 1, 4 and 7, L-phenylalanyl–$tRNA_{CUA}$; lanes 2, 5 and 8, β-(*p*-bromophenyl)alanyl–$tRNA_{CUA}$; lanes 3, 6 and 9, unacylated $tRNA_{CUA}$. The suppression efficiency relative to L-phenylalanine is shown below each lane. Lanes 1–3, modification of position 10 in DHFR; lanes 4–6, modification of position 18 in DHFR; lanes 7–9, modification of position 49 in DHFR.

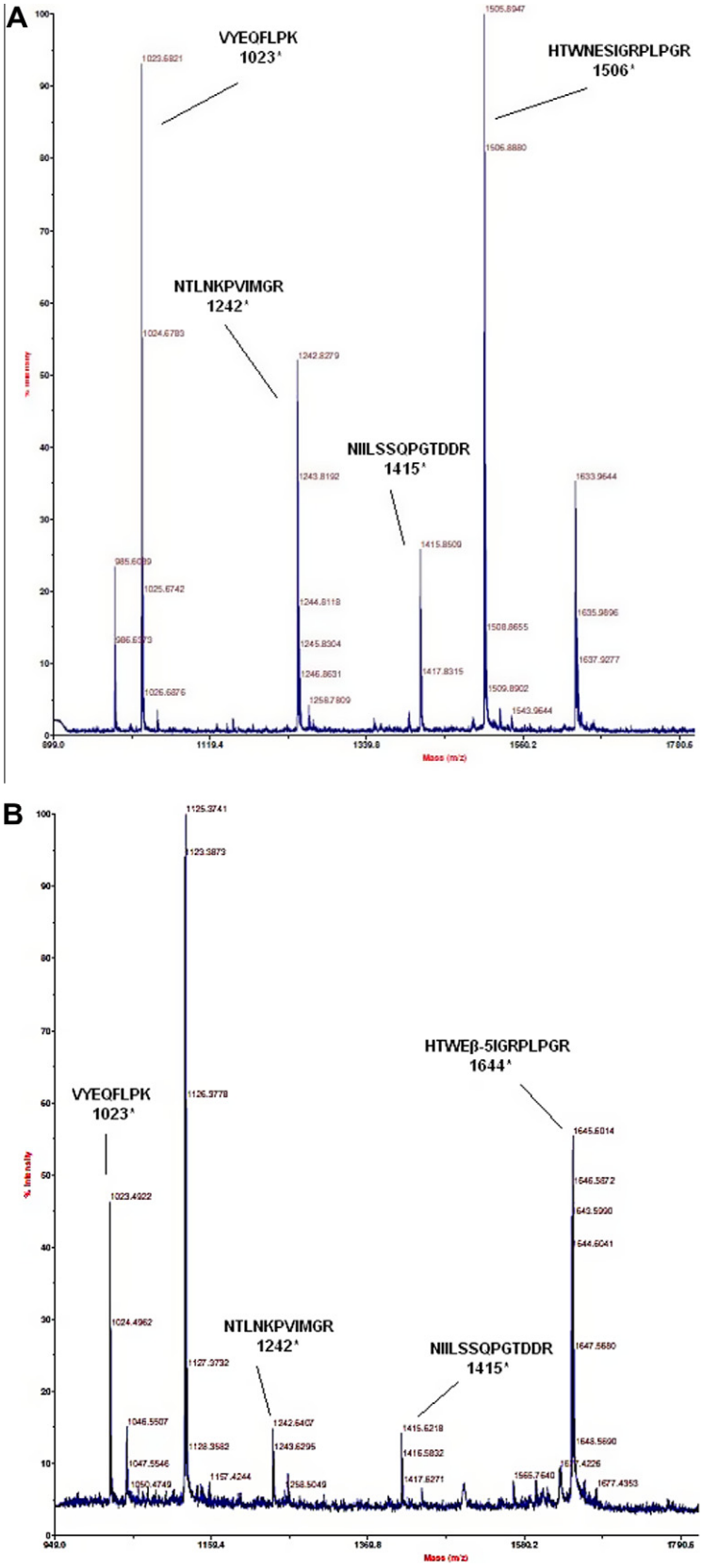


Figure 4. MALDI-MS of tryptic fragments of wild-type (**A**) and modified DHFRs (**B**), the latter having β-amino acid **5** in position 49. Mass range 1800–2500 Da (* = estimated value in Da).

phenyl)alanyl–$tRNA_{CUA}$, putatively affording modified DHFR 1 containing β-amino acid **5** at position 49 (Table 4). The second employed pETDH18 and β,β-dimethyl-β-alanyl–$tRNA_{CUA}$, affording modified DHFR 2 believed to have β-amino acid **3** at position 18 (Table 4). Both modified DHFRs were purified by the use of Ni-NTA chromatography, followed by purification on DEAE–Sephadex

Table 4
MALDI analysis of tryptic digests of wild-type and modified DHFR samples

Position	Peptide sequence	MALDI-MS analysis, molecular mass, Da					
		Wild-type		Modified DHFR 1		Modified DHFR 2	
		Est	MS	Est	MS	Est	MS
13–32	VIGMENAMPWNLPADLAWFK	2304	2304.1	2304	2303.6	**2289**	**2289.9**
34–44	NTLNKPVIMGR	1242	1242.8	1242	1242.6	1242	1242.8
45–57	HTWESIGRPLPGR	1506	1505.8	**1644**	**1645.6**	1506	1505.9
59–71	NIILSSQPGTDDR	1415	1415.8	1415	1415.6	1415	1415.8
72–76	VTWVK	632	632.5	632	634.3	632	632.4
77–98	VDEAIAASGDVPEIMVIGGGR	2028	2029.1	2028	2029.7	2028	2030.7
99–106	VYEQFLPK	1023	1023.7	1023	1023.5	1023	1023.6

Modified DHFR 1: β-alanine analogue **5** in position 49; modified DHFR 2: β-alanine analogue **3** in position 18. Positions 18 and 49 are indicated in red.
The values in boldface reflect the presence of the modified β -amino acids.

and then SDS–polyacrylamide gel electrophoresis. Samples of the DHFR were treated with trypsin according to a published 'in-gel' protocol[21]and the resulting peptide fragments were subjected to MALDI analysis. A sample of wild-type DHFR was used as a control in MALDI analysis for both modified DHFRs. The molecular weights of the tryptic peptides from the wild-type and modified DHFRs were compared (Table 4). In the case of the modified DHFR 1 having β-amino acid **5** at position 49, a tryptic fragment encompassing amino acids 45–57 was anticipated to have a molecular mass of 1644. As shown in Table 4 and Figure 4, there was an ion peak observed *m*/*z* 1644 having a characteristic bromine isotope pattern. This ion was not present in the tryptic digest of wild-type DHFR, but the anticipated ion at *m*/*z* 1506 (reflecting the presence of Ser49) was readily apparent. The other six tryptic peptides encompassing amino acids 13–106 of DHFR were all observed in both the wild-type and modified DHFRs, and gave ion peaks at the same molecular masses.[22] Similarly, the modified DHFR 2 having β-amino acid **3** incorporated at position 18 gave an ion peak at *m*/*z* 2289.9 (estimated value 2289) corresponding to a peptide encompassing amino acids 13–32 (Table 4 and Supplementary data, Fig. 3). This peak was not present in the tryptic digest of the wild-type DHFR, but the anticipated ion peak at *m*/*z* 2304 (reflecting the presence of Asn18) was present. Again, the remaining six tryptic peptides in the wild-type and modified DHFRs were all observed and gave the same ion peaks.

2.6. GluC digestion probing of DHFR structure

The proteolytic stability of β-peptides has been studied in detail by Seebach and co-workers[6] through the use of a set of 15 different proteases, including the commercially available serine proteases trypsin (Arg and Lys in P1 position) and chymotrypsin (Tyr, Phe, Trp and Leu in P1 position). Peptides containing β^2, $\beta^{2,2}$, $\beta^{2,3}$, $\beta^{2,2,3}$ and $\beta^2\beta^3$ substituted amino acids were studied and it was shown that none of the proteases was able to degrade the β-amino acid containing species. There is presently no direct evidence that the presence of a β-amino acid adjacent to a protease cleavage site in a protein would actually affect protease cleavage. However, it is logical to anticipate that cleavage would be impaired, and we utilized DHFR 2, putatively containing β-amino acid **3** in position 18, to study this possibility. DHFR has a glutamic acid at position 17, and we have shown previously that GluC endoproteinase (which hydrolyzes peptide bonds at the C-terminus of glutamic acid residues[23]) hydrolyzes DHFR between Glu17 and Asn18.[24] Shown in Figure 5 is a polyacryalamide gel used to analyze the hydrolysis products resulting from treatment of wild-type and modified DHFRs with GluC endoproteinase. Although hydrolytic cleavage was not complete under the conditions employed, a comparison of lanes 1 and 2 shows that GluC endoproteinase produced bands from wild-type DHFR corresponding to fragments having molecular

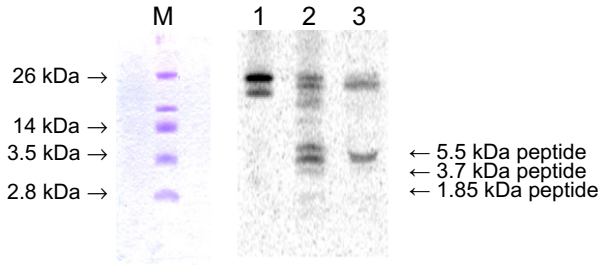


Figure 5. GluC endoproteinase digestion of modified DHFR sample. Lane 1, non-digested DHFR; lane 2, digested sample of wild-type DHFR; lane 3, digested sample of DHFR having β-amino acid **3** in position 18; lane M, molecular weight marker.

weights of 3.7 and 1.85 kDa, as would be expected for the peptides **M**ISLIAALAFDRVIG**M**E and NA**M**PWNLPADLAWFKRNTLNKPVI **M**GRHTWE, resulting from hydrolysis at Glu17 and Glu48. In comparison, analogous digestion of the modified DHFR putatively having β-amino acid **3** at position 18 afforded only a single radioactive peptide (5.5 kDa) (lane 3). This peptide likely resulted from failed digestion following Glu17, resulting in the appearance of the peptide MISLIAALAFDRVIGME3AMPWNLPADLAWFKRNTLNKPVIMGRHTWE instead of the two peptides produced from wild-type DHFR. This observation provides further evidence for the presence of β-amino acid **3** at position 18 of modified DHFR 2. It also suggests that proteins containing β-amino acids at specific positions may be expected to exhibit enhanced protease resistance in proximity to those positions, a finding of potential importance to the design of proteins for therapeutic and other uses.[25]

3. Conclusions

Four β-amino acids having quite different substitution patterns, have been used to activate a suppressor tRNA transcript, and were then incorporated into each of three positions in DHFR by suppression of UAG codons engineered into specific positions in DHFR mRNA. The incorporation was mediated by S-30 preparations containing ribosomes modified in the peptidyltransferase center, and selected for their ability to recognize β-amino acids. The suppression yields, which depended on the specific modified ribosomes employed, as well as the structures of the individual amino acids, ranged up to 18% and were entirely sufficient to enable the preparation of modified DHFRs in amounts sufficient for routine biochemical studies. The incorporation of selected β-amino acids was verified following trypsin digestion by mass spectrometric analysis of the derived peptide fragments, and was limited in each case analyzed to the intended fragment. β-Amino acids have been shown to impart specific conformational biases to peptides into which they have been incorporated,[1–3] and it is anticipated that

analogous effects will be observed for proteins containing β-amino acids. This may be of great advantage in stabilizing protein structures conformationally, especially where multiple folded conformations are substantially populated. The ability of such amino acids to stabilize proteins from proteolytic degradation was suggested by the stability of a DHFR containing β,β-dimethyl-β-alanine (**3**) at position 18 to GluC endoproteinase-mediated cleavage following a glutamic acid residue at position 17.

4. Experimental

4.1. General materials and methods

Reagents and solvents for chemical synthesis were purchased from Aldrich Chemical Co. or Sigma Chemical Co. and were used without further purification. Compounds **4** and **5** were purchased from Peptech Corporation and were used without further purification. All reactions involving air- or moisture-sensitive reagents or intermediates were performed under argon. Analytical TLC was performed using Silicycle silica gel 60 Å F254 plates (0.25 mm), and was visualized by UV irradiation (254 nm). Flash chromatography was performed using Silicycle silica gel (40–60 mesh). ^{1}H and ^{13}C NMR spectra were obtained using a Varian 400 MHz NMR spectrometer. Chemical shifts are reported in parts per million (ppm, δ) referenced to the residual ^{1}H of the solvent ($CDCl_3$, δ 7.26). ^{13}C NMR spectra were referenced to the residual ^{13}C resonance of the solvent ($CDCl_3$, δ 77.16). Splitting patterns are designated as follows: s, singlet; d, doublet; q, quartet; m, multiplet. High resolution mass spectra were obtained at the Arizona State University CLAS High Resolution Mass Spectrometry Facility or the Michigan State University Mass Spectrometry Facility.

Tris, acrylamide, bis-acrylamide, urea, ammonium persulfate, *N*,*N*,*N*′,*N*′-tetramethylenediamine (TEMED), dihydrofolic acid, glycerol, ampicillin, pyruvate kinase, lysozyme, erythromycin**,** isopropyl-β-D-thiogalactopyranoside (IPTG), dithiothreitol (DTT) and 2-mercaptoethanol were purchased from Sigma Chemicals (St. Louis, MO). ^{35}S-Methionine (10 μCi/μL) was obtained from Amersham (Pitscataway, NJ). BL-21 (DE-3) competent cells and T4 RNA ligase were from Promega (Madison, WI). Plasmid MaxiKit (Life Science Products, Inc., Frederick, CO) and GenElute^{TM}HP plasmid miniprep kit (Sigma) were used for plasmid purification.

Phosphorimager analysis was performed using a Molecular Dynamics 400E PhosphorImager equipped with ImageQuant version 3.2 software. Ultraviolet and visible spectral measurements were made using a Perkin-Elmer lambda 20 spectrophotometer.

4.2. Synthesis and characterization of aminoacylated pdCpA derivatives

4.2.1. *N*-4-Pentenoyl-*β*-*S*-(*p*-bromophenyl)alanine cyanomethyl ester (6)[16]

To a solution containing 100 mg (0.41 mmol) of **5** and 70.0 mg (0.82 mmol) of $NaHCO_3$ in 4 mL of 1:1 dioxane/H_2O was added 90 mg (0.45 mmol) of 4-pentenoyloxysuccinimide. The reaction mixture was stirred at room temperature for 24 h under argon. The reaction was quenched by the addition of 15 mL of 1 N aq $NaHSO_4$ and the aqueous layer was extracted with two 25-mL portions of ethyl acetate. The combined organic extract was dried ($MgSO_4$) and concentrated to dryness under diminished pressure. The crude product was then dissolved in 4 mL of acetonitrile. To this solution were added 300 μL (2.10 mmol) of triethylamine and 130 μL (2.10 mmol) of chloroacetonitrile. The reaction mixture was stirred at room temperature for 24 h at which time 20 mL of ethyl acetate was added. The organic layer was washed with 10 mL of 1 N aq $NaHSO_4$ followed by 10 mL of brine, then dried ($MgSO_4$) and concentrated to dryness under diminished pressure, affording a crude residue. The crude product was purified on a flash silica gel column (15 × 1 cm) using 1:1 ethyl acetate/hexane for elution to obtain **6** as a colorless semi-solid: yield 140 mg (38%); R_f 0.33 (1:1 ethyl acetate/hexane); ^{1}H NMR ($CDCl_3$) δ 2.27–2.40 (m, 4H), 2.85–3.03 (m, 2H), 4.65 (s, 2H), 4.99–5.07 (m, 2H), 5.39 (q, 1H, *J* = 6.8 Hz), 5.74–5.84 (m, 1H), 6.45 (d, 1H, *J* = 8.0 Hz), 7.15 (d, 2H, *J* = 8.0 Hz), and 7.45–7.47 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 29.4, 35.6, 39.2, 48.6, 49.0, 113.9, 115.9, 121.9, 128.1, 132.0, 136.7, 138.8, 169.3, and 171.8; mass spectrum (APCI), *m*/*z* 365.0631 $(M+H)^+$ ($C_{17}H_{20}NO_3Br$ requires *m*/*z* 365.0627).

4.2.2. *N*-4-Pentenoyl-*β*-*S*-(*p*-bromophenyl)alanyl-pdCpA (8)[16]

To a 1.5-mL tube containing 5.3 mg (3.7 μmol) of the tris(tetrabutylammonium) salt of pdCpA[20] (TBA–pdCpA, **7**) was added 10 mg (27 μmol) of **6** dissolved in 100 μL of 9:1 DMF/Et_3N. After 2 h of sonication, the reaction mixture was purified by C_{18} reversed phase HPLC using a gradient of 1%→65% acetonitrile in 50 mM ammonium acetate, pH 4.5, over a period of 45 min. The fractions eluting at 20.8 and 22.1 min were collected, combined and lyophilized to afford a colorless solid: yield 3.1 mg (100%); mass spectrum (ESI), *m*/*z* 942.1234 $(M-H)^-$ ($C_{33}H_{39}N_9O_{15}BrP_2$ requires *m*/*z* 942.1230).

4.2.3. Preparation and characterization of the pdCpA esters of amino acids 2, 3 and 4

The pdCpA esters of the remaining three amino acids (**2**, **3** and **4**) were prepared in the same fashion as the synthesis of **8** described above. Each was purified by C_{18} reversed phased HPLC (Supplementary data, Fig. 4) and afforded satisfactory high resolution mass spectrometric data as follows:

4.2.3.1. *N*-4-Pentenoyl-3-amino-(*R*,*S*)-2-methylpropionyl-pdCpA (8a). Mass spectrum (ESI), *m*/*z* 802.1977 $(M-H)^-$ ($C_{28}H_{38}N_9O_{15}P_2$ requires *m*/*z* 802.1968).

4.2.3.2. *N*-4-Pentenoyl-3-amino-3,3-dimethylpropionyl-pdCpA (8b). Mass spectrum (ESI), *m*/*z* 816.2130 $(M-H)^-$ ($C_{29}H_{40}N_9O_{15}P_2$ requires *m*/*z* 816.2125).

4.2.3.3. *N*-4-Pentenoyl-β-*S*-phenylalanyl-pdCpA (8c). Mass spectrum (ESI), *m*/*z* 864.2126 $(M-H)^-$ ($C_{33}H_{40}N_9O_{15}P_2$ requires *m*/*z* 864.2125).

4.3. β-Aminoacyl–tRNA$_{CUA}$s

The activation of suppressor tRNA$_{CUA}$s was carried out as described previously.[16] Briefly, 100-μL reaction mixtures of 100 mM Na Hepes, pH 7.5, contained 1.0 mM ATP, 15 mM $MgCl_2$, 100 μg of suppressor tRNA$_{CUA}$-C$_{OH}$, 0.5 A_{260} unit of *N*-pentenoyl-protected β-aminoacyl-pdCpA, 15% DMSO, and 100 units of T4 RNA ligase. The reaction mixture was incubated at 37 °C for 1 h, then quenched by the addition of 0.1 vol of 3 M NaOAc, pH 5.2. The N-protected aminoacylated tRNA was precipitated with 3 vol of cold ethanol. The efficiency of ligation was estimated by 8% polyarylamide–7 M urea gel electrophoresis (pH 5.0).[26]

The *N*-pentenoyl-protected aminoacyl–tRNA$_{CUA}$s were deprotected by treatment with 5 mM aqueous I_2 at 25 °C for 15 min. The solution was centrifuged, and the supernatant was adjusted to 0.3 M NaOAc and treated with 3 vol of cold ethanol to precipitate the aminoacylated tRNA. The tRNA pellet was washed with 70% aq EtOH, air dried and dissolved in 50 μL of RNase free water.

4.4. Preparation of S-30 extracts from cells having modified ribosomes

Aliquots (5–10 μL) from liquid stocks of *E. coli* BL-21(DE-3) cells, harboring plasmids with a wild-type or modified rrnB gene, were placed on LB agar supplemented with 100 μg/mL of ampicillin and grown at 37 °C for 16–18 h. One colony was picked from each agar plate and transferred into 3 mL of LB medium supplemented with 100 μg/mL of ampicillin and 0.5 mM IPTG. The cultures were grown at 37 °C for 3–6 h in a thermostated shaker until OD_{600} ~0.15–0.3 was reached (about 3–5 h), diluted with LB medium supplemented with 100 μg/mL ampicillin, 1 mM IPTG and 3 μg/mL of erythromycin (for selectively enhancing the modified ribosome fraction) until OD_{600} 0.01 was reached, and then grown at 37 °C for 12–18 h. The optimal concentration of the final cultures was OD_{600} 0.5–1.0. Cells were harvested by centrifugation (5000×*g*, 4 °C, 10 min), washed three times with S-30 buffer (1 mM Tris–OAc, pH 8.2, containing 1.4 mM $Mg(OAc)_2$, 6 mM KOAc and 0.1 mM DTT) supplemented with β-mercaptoethanol (0.5 mL/L) and once with S-30 buffer having 0.05 mL/L β-mercaptoethanol. The weight of the wet pellet was estimated and 1.27 mL of S-30 buffer was added to suspend 1 g of cells. The volume of the suspension was measured and used for estimating the amount of other components. Pre-incubation mixture (0.3 mL) (0.29 M Tris, pH 8.2, containing 9 mM $Mg(OAc)_2$, 13 mM ATP, 84 mM phosphoenol pyruvate, 4.4 mM DTT and 5 μM amino acids mixture), 15 units of pyruvate kinase and 10 μg of lyzozyme were added per 1 mL of cell suspension and the resulting mixture was incubated at 37 °C for 30 min. The incubation mixture was then frozen at −80 °C (~30 min), melted (37 °C, 30 min), and again frozen and melted at room temperature (~30 min). Ethylene glycol tetraacetic acid (EGTA) was then added to 2.5 mM final concentration and the cells were incubated at 37 °C for 30 min and again frozen (−80 °C, 30 min). The frozen mixture was centrifuged (15,000×*g*, 4 °C, 1 h) and the supernatant was stored in aliquots at −80 °C.

4.5. In vitro protein translation

Protein translation reactions were carried out in 12–2000 μL of incubation mixture containing 0.2–0.4 μL/μL of S-30 system, 100 ng/μL of plasmid, 35 mM Tris acetate, pH 7.4, 190 mM potassium glutamate, 30 mM ammonium acetate, 2 mM DTT, 0.2 mg/mL total *E. coli* tRNA, 3.5% PEG 6000, 20 μg/mL folinic acid, 20 mM ATP and GTP, 5 mM CTP and UTP, 100 μM amino acids mixture, 0.5 μCi/μL of ^{35}S-methionine and 1 μg/mL rifampicin. In the case of plasmids having a gene with a TAG codon, a suppressor tRNA was added to a concentration of 0.3–0.5 μg/μL (for α-aminoacyl-tRNAs) and 0.6–1.0 μg/μL (for β-aminoacyl-tRNAs). Reactions were carried out at 37 °C for 1 h and terminated by chilling on ice. Aliquots from in vitro translation mixtures were analyzed by SDS–PAGE followed by quantification of the radioactive bands by phosphorimager analysis.

4.6. Purification of DHFR

Samples of DHFR, prepared during in vitro translation, were diluted with 50 mM Na phosphate, pH 8.0, containing 0.3 M NaCl and 0.1 mg/ml BSA and applied to a 50-μL Ni-NTA agarose column that had been equilibrated with the same buffer. The column was washed with three 500-μL portions of the same buffer and DHFR was eluted by washing with 150 μL of the same buffer also containing 250 mM imidazole.

A 50-μL column of DEAE-Sepharose was equilibrated with 500 μL of 10 mM Na phosphate, pH 7.4. Samples of DHFR purified by Ni-NTA chromatography were diluted 10-fold in the same buffer and applied to the resin. The column was washed with 500 μL of the same buffer. DHFR was eluted with a NaCl step gradient (0.1; 0.2; 0.3; 0.4; 0.5 M) in 10 mM Na phosphate, pH 7.4. The fractions were analyzed on 12% polyacrylamide gel, stained using Coomassie R-250, then destained in 20% ethanol/7% acetic acid. The fractions containing DHFR were combined, dialyzed against 50 mM Na phosphate, pH 7.4, and concentrated by ultrafiltration using a YM-10 filter (Amicon Ultra, Millipore Corp, Billerica, MA).

4.7. 'In gel' trypsin digestion

Samples to be digested in the gel were run in 3–4 lanes of a 12% SDS–polyacrylamide gel, stained with Coomassie R-250 and destained until the background was clear. That area of the gel having the DHFR was cut from the gel and washed with 0.1 M ammonium bicarbonate (1 h, room temperature). The solution was discarded and 0.1–0.2 mL of 0.1 M ammonium bicarbonate and 10–30 μL of 0.045 mM DTT were added. Gel pieces were incubated at 60 °C for 30 min, cooled to room temperature and incubated at room temperature for 30 min in the dark after the addition of 10–30 μL of 0.1 M iodoacetamide. Gel pieces were washed in 50% acetonitrile/0.1 M ammonium bicarbonate until they became colorless. After discarding the solution, the gel pieces were incubated in 0.1–0.2 mL of acetonitrile (10–20 min at room temperature) and, after removal of solvent, were re-swelled in 50–100 μL of 25 mM ammonium bicarbonate containing 0.02 μg/μL trypsin. After incubation at 37 °C for 4 h, the supernatant was removed to a new tube and the peptides were extracted with 60% acetonitrile/0.1% TFA (20 min at room temperature). The combined fractions were dried and reconstituted in minimum amount of 60% acetonitrile/0.1% TFA.

4.8. GluC endoproteinase digestion

DHFR samples radiolabeled by in vitro translation in the presence of ^{35}S-methionine were purified by successive chromatographic purification of Ni-NTA and DEAE-Sepharose columns. The samples were transferred to 50 μL of 50 mM Tris–HCl, pH 8.0, containing 0.5 mM Glu–Glu, by the use of a YM-10 ultrafiltration device (Amicon Ultra, Millipore Corp, Billerica, MA). The radioactivity in the samples was checked by liquid scintillation counting. About 20 ng of each DHFR sample (~10^7cpm) was mixed with 2 μg of GluC endoproteinase and incubated at 25 °C for 16 h. The total reaction volume was 20 μL. Reactions were quenched by the addition of 2 μL of 1.1% formic acid and the final samples (5–10 μL) were analyzed by 20% Tris–tricine polyacrylamide gel electrophoresis[27] followed by analysis using a phosphorimager.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.01.002.

References and notes

1. Rohl, C. A.; Chakrabartty, A.; Baldwin, R. L. *Protein Sci.* **1996**, *5*, 2623.
2. Blaber, M.; Zhang, X. J.; Lindstrom, J. D.; Pepiot, S. D.; Baase, W. A.; Matthews, B. W. *J. Mol. Biol.* **1994**, *235*, 600.
3. Park, S. H.; Shalongo, W.; Stellwagen, E. *Biochemistry* **1993**, *32*, 7048.
4. Hart, S. A.; Bahadoor, A. B. F.; Matthews, B. W.; Qiu, X. Y. J.; Schepartz, A. *J. Am. Chem. Soc.* **2003**, *125*, 4022.
5. Cheng, R. P.; DeGrado, W. F. *J. Am. Chem. Soc.* **2001**, *123*, 5162.
6. Frackenpohl, J.; Arvidsson, P. I.; Schreiber, J. V.; Seebach, D. *ChemBioChem* **2001**, *2*, 445.
7. Appella, D. H.; Christianson, L. A.; Klein, D. A.; Powell, D. R.; Huang, X. L.; Barchi, J. J.; Gellman, S. H. *Nature* **1997**, *387*, 381.
8. Seebach, D.; Gardiner, J. *Acc. Chem. Res.* **2008**, *41*, 1366.
9. Kritzer, J. A.; Stephens, O. M.; Guarracino, D. A.; Reznik, S. K.; Schepartz, A. *Bioorg. Med. Chem.* **2005**, *13*, 11.

10. Porter, E. A.; Wang, X.; Lew, H.-S.; Weisblum, B.; Gellman, S. H. *Nature* **2000**, *404*, 565.
11. Cristau, M.; Devin, C.; Oiry, C.; Chaloin, O.; Amblard, M.; Bernad, N.; Heitz, A.; Fehrentz, J.-A.; Martinez, J. *J. Med. Chem.* **2000**, *43*, 2356.
12. Heckler, T. G.; Roesser, J. R.; Xu, C.; Chang, P.-I.; Hecht, S. M. *Biochemistry* **1988**, *27*, 7254.
13. Roesser, J. R.; Xu, C.; Payne, R. C.; Surratt, C. K.; Hecht, S. M. *Biochemistry* **1989**, *28*, 5185.
14. Dedkova, L. M.; Fahmi, N. E.; Golovine, S. Y.; Hecht, S. M. *J. Am. Chem. Soc.* **2003**, *125*, 6616.
15. Dedkova, L. M.; Fahmi, N. E.; Golovine, S. Y.; Hecht, S. M. *Biochemistry* **2006**, *45*, 15541.
16. Dedkova, L. M.; Fahmi, N. E.; Paul, R.; del Rosario, M.; Zhang, L.; Chen, S.; Feder, G.; Hecht, S. M. *Biochemistry* **2012**, *51*, 401.
17. Lodder, M.; Golovine, S.; Hecht, S. M. *J. Org. Chem.* **1997**, *62*, 778.
18. Lodder, M.; Golovine, S.; Laikhter, A. L.; Karginov, V. A.; Hecht, S. M. *J. Org. Chem.* **1998**, *63*, 794.
19. Robertson, S. A.; Ellman, J. A.; Schultz, P. G. *J. Am. Chem. Soc.* **1991**, *113*, 2722.
20. Robertson, S. A.; Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Nucleic Acids Res.* **1989**, *17*, 9649.
21. Huynh, M. L.; Russell, P.; Walsh, B. *Methods Mol. Biol.* **2009**, *519*, 507.
22. In replicate experiments, the N-terminal fragment (containing a hexahistidine fusion peptide) and the (large) C-terminal fragment were never observed.
23. Drapeau, G. R. *Methods Enzymol.* **1976**, *45*, 469.
24. Karginov, V. A.; Mamaev, S. V.; An, H.; Van Cleve, M. D.; Hecht, S. M.; Komatsoulis, G. A.; Abelson, J. N. *J. Am. Chem. Soc.* **1997**, *119*, 8166.
25. Kamionka, M. *Curr. Pharm. Biotechnol.* **2011**, *12*, 268.
26. Varshney, U.; Lee, C. P.; RajBhandary, U. L. *J. Biol. Chem.* **1991**, *266*, 24712.
27. Schagger, H.; Aguila, H.; Von Jagow, G. *Anal. Biochem.* **1988**, *173*, 201.