Probing the Role of an Active Site Aspartic Acid in Dihydrofolate Reductase

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Abstract: Analogues of *E. coli* dihydrofolate reductase (DHFR) containing modified amino acids at single, predetermined sites have been prepared. This was accomplished by the use of the DHFR gene containing an engineered nonsense codon (TAG) at the positions corresponding to Val-10 and Asp-27. Misacylated suppressor tRNAs activated with the modified amino acids of interest were employed for the suppression of the nonsense codons in a cell free protein biosynthesizing system, thereby permitting the elaboration of the desired protein analogues. In this fashion, the aspartic acid analogues *erythro*-carboxyproline, cysteic acid, *β*,*β*-dimethylaspartic acid, α-methylaspartic acid, *erythro*- and *threo*-*β*-methylaspartic acid, *N*-methylaspartic acid, and phosphonoalanine were incorporated into one or both of the aformentioned positions. Although a number of these analogues were incorporated only in low yield, a modification of the strategy has suggested how this might be improved significantly. The derived proteins were purified and then characterized by their mobility on polyacrylamide gels in comparison with wild-type DHFR. Representative DHFRs modified at position 10 were also degraded by defined proteolysis with Glu-C endoproteinase; the fragments containing the modified amino acids were shown to have the same chromatographic properties on reverse phase HPLC as authentic synthetic standards. Individual analogues were assayed for their abilities to bind to the substrate analogue methotrexate and to convert dihydrofolate to tetrahydrofolate. DHFR analogues containing *erythro*- and *threo*-*β*-methylaspartic acid and *β*,*β*-dimethylaspartic acid were all shown to mediate tetrahydrofolate production 74−86% as efficiently as wild-type DHFR under conditions of multiple substrate turnover. Analysis of the rates of tetrahydrofolate production in the presence of NADPH and NADPD at two pH values suggests that this was due to rate-limiting hydride transfer from NADPH bound to DHFR analogues whose active site had been altered structurally.

Dihydrofolate reductase ((DHFR) catalyzes the NADPH-dependent reduction of 7,8-dihydrofolic acid to 5,6,7,8-tetrahydrofolic acid. Because the latter species is further converted to N^5,N^{10}-methylenetetrahydrofolic acid, an essential cofactor for 15 one-carbon transfers, DHFR is an enzyme of central importance in the metabolism of virtually all organisms.[1] The enzyme has long been a target for antitumor and antiinfective therapy, and numerous inhibitors have been described.[2]

Likewise, extensive structural and mechanistic analyses of DHFR have been carried out, mostly with the *E. coli* enzyme. Site-directed mutagenesis, NMR spectroscopy, and X-ray crystallography have contributed importantly to a rather detailed understanding of enzyme structure and function.[3] In particular, these studies have revealed that the active sites of all DHFRs contain an amino acid with a carboxylate substituent, Asp-27 in the case of *E. coli* DHFR, that interacts directly with the dihydrofolic acid substrate and plays a critical role in proton donation during catalysis.[4]

We have previously described the synthesis of *E. coli* DHFR in a cell free system that was amenable to overexpression, and which produced wild-type DHFR shown to be fully competent catalytically.[5] Herein, we demonstrate that by introducing a nonsense (UAG) codon at a specific position in the DHFR mRNA, it is possible to elaborate modified DHFRs by inclusion of misacylated suppressor $tRNA_{CUA}$s in the protein biosynthesizing system (Scheme 1). Presently, we describe the synthesis of seven novel DHFRs containing aspartic acid analogues at position 27. The ability of selected DHFR analogues to bind to methotrexate and mediate the reduction of dihydrofolate are described. Three analogues containing bulky aspartic acid

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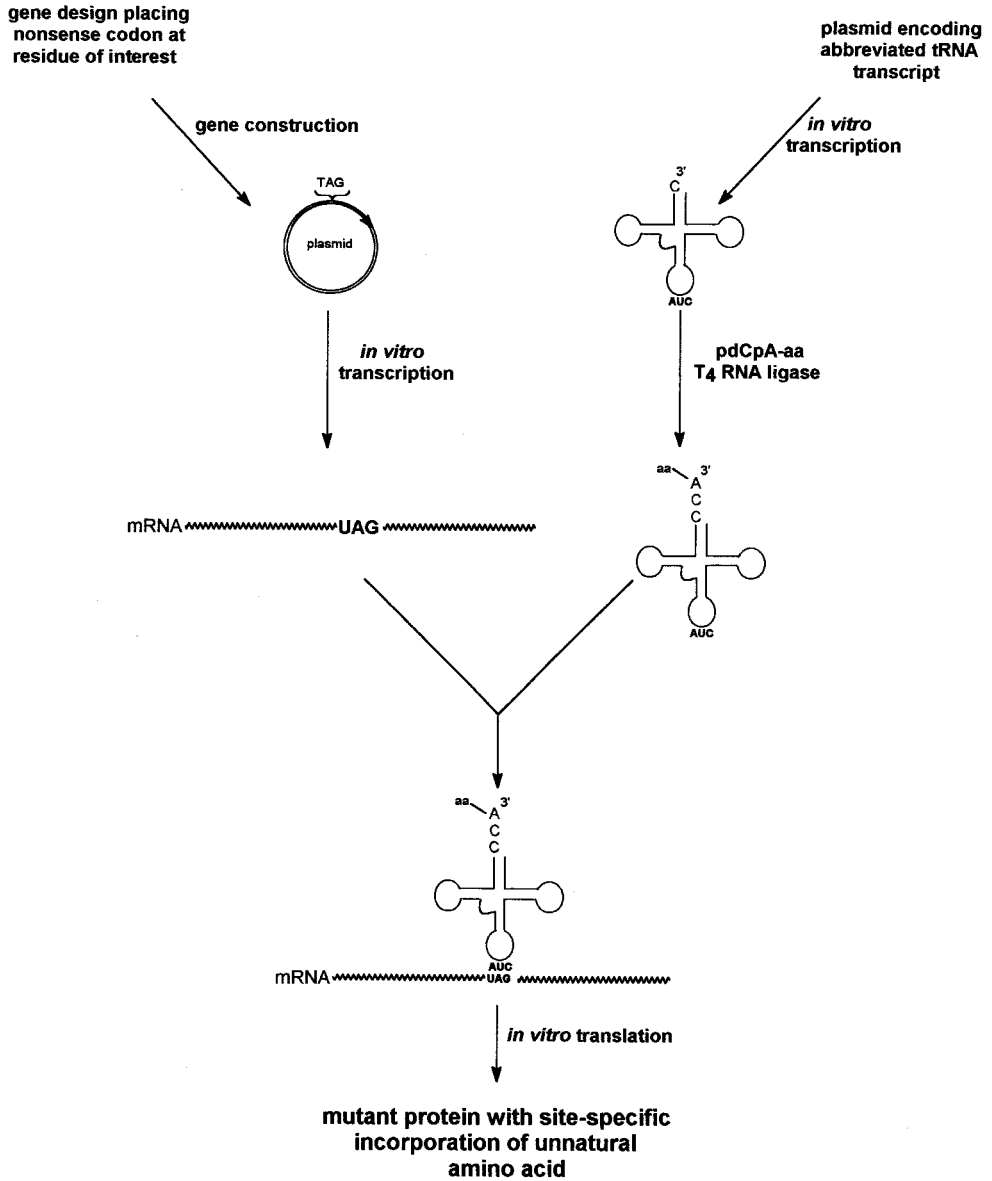
Scheme 1. General Strategy Employed for the Synthesis of Proteins Containing Synthetic Amino Acids at Single, Predetermined Sites



analogues are shown to exhibit diminished rates of tetrahydrofolate production; studies with the DHFR containing β,β-dimethylaspartate at position 27 indicated that this was due to alteration of the rate of hydride transfer.

Results

The elaboration of a number of analogues of *E. coli* dihydrofolate reductase modified at single, predetermined sites has been effected using the general strategy shown in Scheme 1. In this specific case, messenger RNAs containing nonsense (UAG) codons at position 10 or 27 were translated in a cell free protein synthesizing system that employed rabbit reticulocyte lysate (Scheme 2). Translation of these mRNAs to afford full length protein was dependent on the presence of an aminoacylated suppressor tRNA. The derived dihydrofolate reductase analogues were purified to homogeneity by affinity chromatography and used to (i) verify the incorporation of the modified amino acids by suppression of the nonsense codons, (ii) determine the ability of individual dihydrofolate reductase analogues to bind to the folate antagonist methotrexate and (iii) define the properties of key analogues in mediating the conversion of dihydrofolate → tetrahydrofolate.

Construction of Expression Plasmids and in Vitro Transcription. Plasmid pTZRKE (Figure 1), which encodes the gene for *E. coli* dihydrofolate reductase[6] under the control of a T7 promoter, was modified for this study. The modifications included the introduction of the DNA sequence corresponding to the peptide sequence MIHHHHHHE immediately prior to the initiator codon (plasmid pTHis15, Figure 2). The inclusion of the hexahistidine moiety was intended to facilitate purification of the derived DHFR by affinity chromatography on Ni-NTA agarose.[7] In addition, the coding sequence of the DHFR gene was altered to include nonsense (TAG) codons at positions 10 (pTZN2H4) or 27 (pTZ27R2H11).

In vitro transcription of each of the four plasmids was carried out using T7 RNA polymerase following linearization of the plasmid DNAs with restriction endonuclease *Bam*HI. The synthesized RNAs were analyzed by polyacrylamide gel electrophoresis and shown to have the expected (~600 nt) lengths.

In Vitro Protein Synthesis. Preparation of the misacylated $tRNA_{CUA}$s employed for readthrough of nonsense codons was accomplished as shown in Scheme 3, by T4 RNA ligase-mediated coupling of *N*-protected 2′(3′)-*O*-aminoacyl-pdCpA derivatives with tRNA transcripts lacking the 3′-terminal cytidine and adenosine moieties. This strategy, first described

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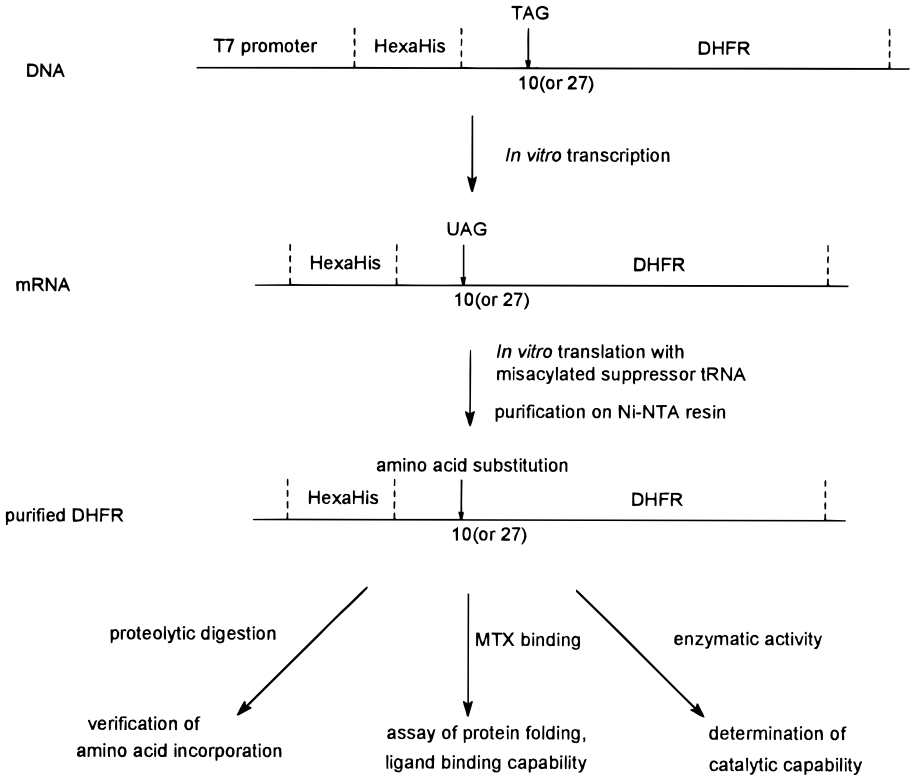
Scheme 2. Strategy Employed for the Elaboration and Characterization of Dihydrofolate Reductases Modifed at Position 10 or Position 27[a]



[a] A linearized plasmid DNA containing the DHFR gene with a stop codon (TAG) at position 10 or 27 was transcribed using T7 RNA polymerase. *In vitro* translation of the derived mRNA in the presence of a misacylated suppressor tRNA afforded DHFR with a modified amino acid at the predetermined site. Following purification on a Ni-NTA resin, the modified DHFRs were assayed for their abilities to bind methotrexate and effect the reduction of dihydrofolate to tetrahydrofolate. Representative DHFRs modified at position 10 were degraded with Glu-C endoproteinase to afford a 17-amino acid polypeptide, the latter of which was compared with an authentic sample for verification of structure.

by Noren et al. in 1989,[8] is a modification of earlier approaches described by the Hecht laboratory[9] and has been studied intensively for more than a decade.[9j,10] The syntheses of the amino acids and aminoacylated pdCpA derivatives employed here will be described elsewhere.[11] Two suppressor tRNA transcripts were tested for efficiency of *in vitro* suppression. One of these, structurally related to yeast $tRNA^{Phe}$, has been described previously;[10d,12] it was obtained for the present study by *in vitro* transcription of a synthetic tRNA gene that had been incorporated into plasmid pUC19 under the control of a T7 promoter. The other tRNA was an *E. coli* $tRNA^{Ala}_{CUA}$ construct containing a one-nucleotide alteration in the acceptor stem that rendered it dysfunctional for aminoacyl-tRNA synthetase-mediated activation.[13] This tRNA was also prepared by *in vitro* transcription from newly constructed plasmid pRHM1. Following activation with valine, both of these tRNAs were employed for the elaboration of dihydrofolate reductase in a rabbit reticulocyte system using an mRNA having the codon UAG at position 10. As shown in Figure 3, both valyl-$tRNA_{CUA}$s displayed the same suppression efficiency in this experiment (~17%) and proved comparable in numerous other tests. The protein synthesizing system employed was carefully

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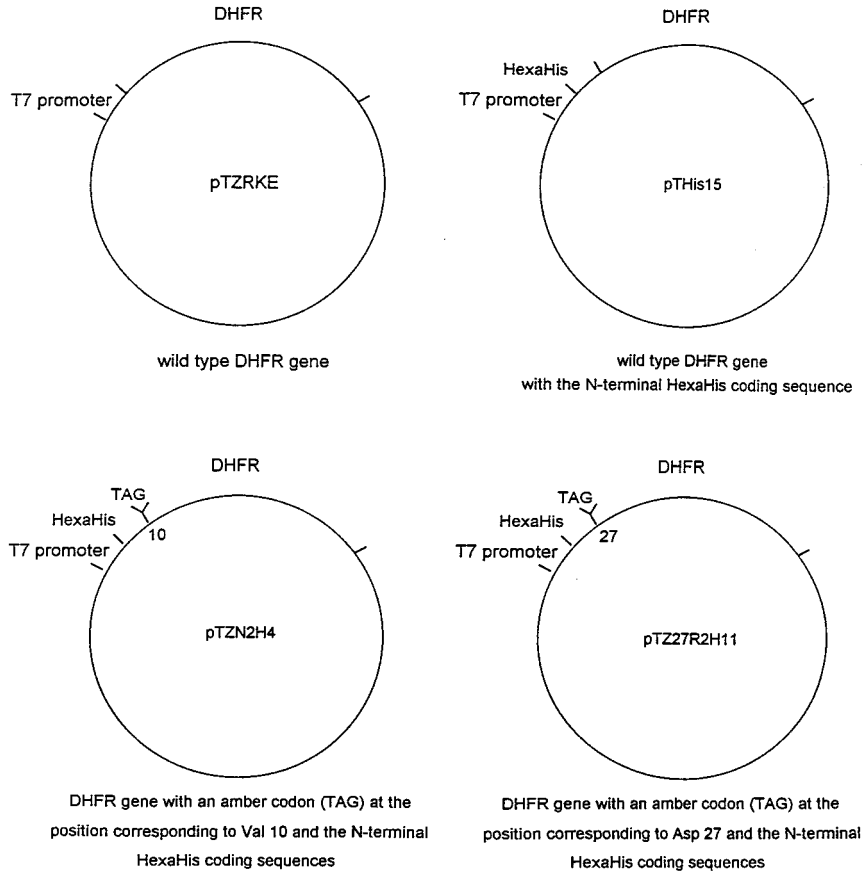
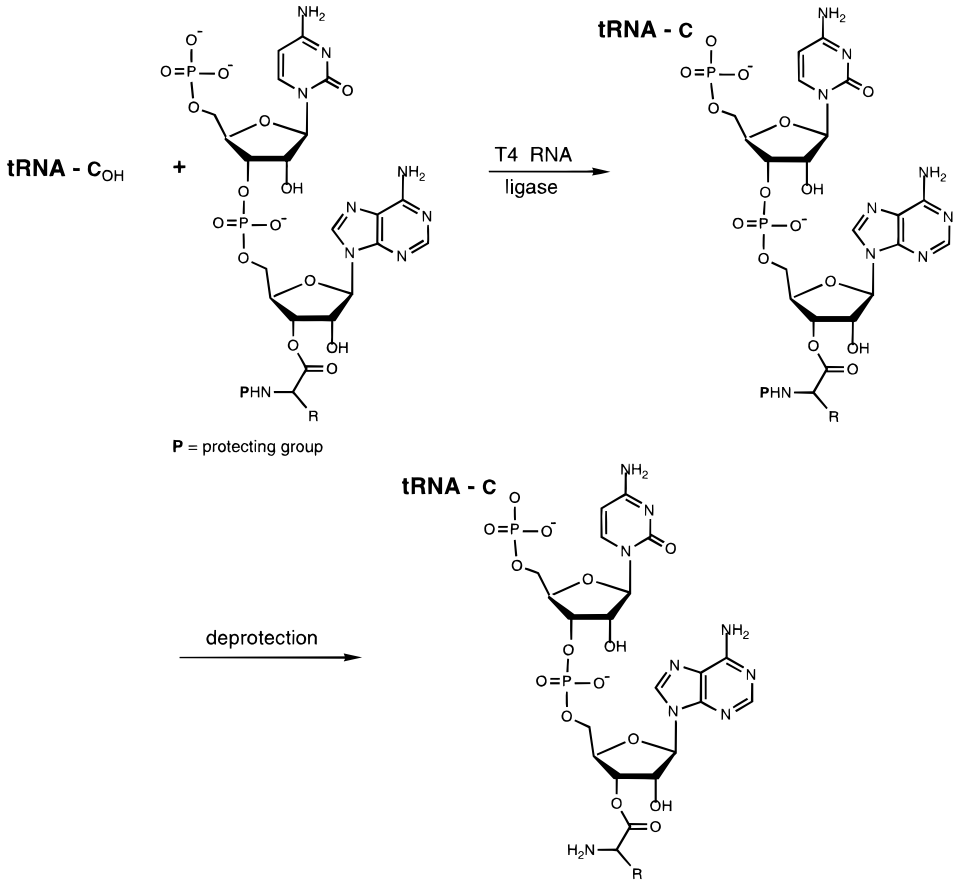


Figure 1. Plasmid constructs used for expression of the DHFR gene.

Scheme 3. "Chemical Aminoacylation" Strategy Employed for the Preparation of Misacylated Suppressor tRNAs



optimized with respect to variables such as concentrations of mRNA, misacylated tRNAs and cofactors, time of incubation, etc. (data not shown).

Elaboration of Modified DHFRs. The preparation of a number of DHFRs modified at position 10 was carried out in an *in vitro* protein biosynthesizing system in the presence of a

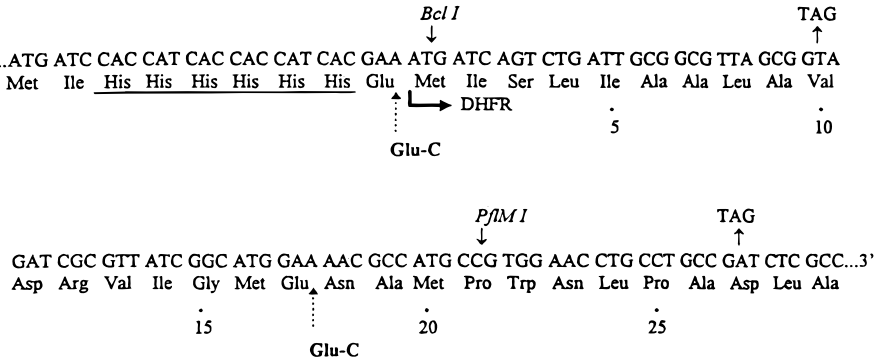


Figure 2. Nucleotide sequence and deduced amino acid sequence at the 5′-end of the DHFR gene. Amino acids are numbered starting from the first Met of wild-type DHFR; a hexahistidine sequence added to the N-terminus to facilitate affinity purification is underlined. Restriction sites are indicated by solid arrows; sites of hydrolysis by Glu-C endoproteinase (Glu-C) by dashed arrows. Also indicated are the sites of introduction of nonsense codons (positions 10 and 27) and the start of the DHFR sequence (bold arrow).

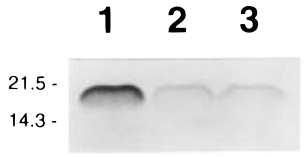


Figure 3. Autoradiogram of a 20% SDS-polyacrylamide gel illustrating the *in vitro* synthesis of [^{35}S]methionine-labeled DHFR: lane 1, pTHis (wild-type) mRNA; lane 2, pTZN2H4 mRNA (UAG at codon 10) + *E. coli* valyl-tRNA$^{Ala}_{CUA}$; and lane 3, pTZN2H4 mRNA + yeast valyl-tRNA$^{Phe}_{CUA}$. The molecular weight observed for the derived protein, as judged from several molecular weight markers (two of which are shown, in kDa), was consistent with the expected value.

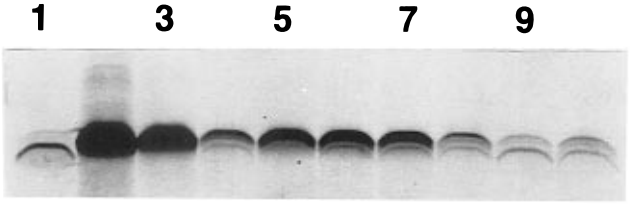


Figure 4. Elaboration of dihydrofolate reductase analogues containing modified amino acids at position 10. Protein synthesis was carried out in the presence of ^{35}S-methionine using a rabbit reticulocyte lysate, mRNA containing UAG at codon position 10 and no suppressor tRNA (lane 1) or a suppressor tRNA activated with one of several amino acids (lanes 3−10). Following incubation at 30 °C for 2 h, the reaction mixtures were analyzed on a 20% SDS-polyacrylamide gel: lane 1, no tRNA$_{CUA}$; lane 2, wild-type mRNA, no tRNA$_{CUA}$; lane 3, valine; lane 4, aspartic acid; lane 5, *β*,*β*-dimethylaspartic acid; lane 6, *erythro*-*β*-methylaspartic acid; lane 7, *threo*-*β*-methylaspartic acid; lane 8, cysteic acid; lane 9, *erythro*-carboxyproline; and lane 10, *N*-methylaspartic acid. The band in lane 1 is believed to represent a translation product initiated at an internal (position 16) AUG codon (cf Figure 2).

mRNA containing a UAG codon at the position to be modified and a suppressor tRNA activated with a synthetic amino acid. As shown in Figure 4, the elaboration of full length protein was dependent on the presence of an aminoacylated tRNA$_{CUA}$; in the absence of this species (lane 1), the derived product was a protein formed by initiation at the internal methionine codon located at position 16 of full length DHFR, presumably formed by protein synthesis termination and reinitiation.[14] Likewise, negligible amounts of full length DHFR were obtained in the presence of unacylated, full length suppressor tRNAs, consistent with published observations for these species.[8,13] In the presence of aminoacylated tRNA$_{CUA}$s, several full length DHFRs were formed,[15] including species containing *β*,*β*-dimethylaspartic acid,

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(15) Admixture of unacylated tRNA$_{CUA}$s to the translation mixtures afforded no DHFR protein because neither suppressor tRNA is a substrate for the endogenous aminoacyl-tRNA synthetases.

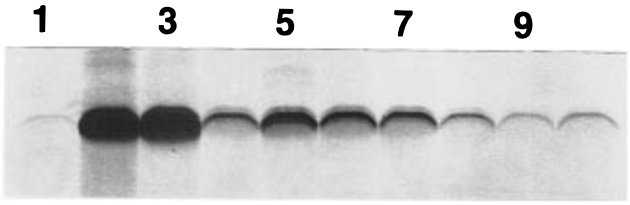

Figure 5. Synthesis of dihydrofolate reductase analogues containing structurally modified amino acids at position 27. Protein synthesis was carried out and analyzed as described in the legend to Figure 4 with the exception that the mRNA contained a UAG codon at position 27 (derived from plasmid pTZ27R2H11): lane 1, no tRNA$_{CUA}$; lane 2, wild-type mRNA, no tRNA$_{CUA}$; lane 3, valine; lane 4, aspartic acid; lane 5, *β*,*β*-dimethylaspartic acid; lane 6, *erythro*-*β*-methylaspartic acid; lane 7, *threo*-*β*-methylaspartic acid; lane 8, cysteic acid; lane 9, *erythro*-carboxyproline; and lane 10, *N*-methylaspartic acid.

Table 1. Suppression Efficiencies by Aminoacyl-tRNA$_{CUA}$ at Positions 10 and 27 of Dihydrofolate Reductase

	suppression efficiency (%)[a]	
amino acid	position 10	position 27
aspartic acid, allyl ester		38
aspartic acid	7	11
erythro-carboxyproline	*b*	3
threo-carboxyproline		*b*
cysteic acid	3	3
β,*β*-dimethylaspartic acid	9	16
α-methylaspartic acid	3	*b*
erythro-*β*-methylaspartic acid	8	7
threo-*β*-methylaspartic acid	11	10
N-methylaspartic acid	4	*b*
phenylalanine	20	15
phosphonoalanine	*b*	3
valine	30	41

[a] Defined as the percentage of DHFR produced via nonsense codon suppression relative to production of DHFR from wild-type mRNA. [b] Incorporation not significantly above background.

erythro- and *threo*-*β*-methylaspartic acid, cysteic acid, and *N*-methylaspartic acid (Figure 4). Quantification of the extent of DHFR formation in the presence of 12 different aminoacyl-tRNA$_{CUA}$s was carried out by phosphorimager analysis and is summarized in Table 1. An analogous set of experiments was carried out to effect the incorporation of 13 modified amino acids into position 27, which normally contains an active site aspartic acid (Figure 5 and Table 1). A few trends are apparent from the data summarized in Table 1. First, there was a substantial variation from one amino acid to the next in the efficiency of incorporation at a given site, indicating that the nature of the amino acid side chain affects the efficiency of nonsense codon readthrough. Nonpolar amino acids such as phenylalanine and valine were incorporated with greater ef-

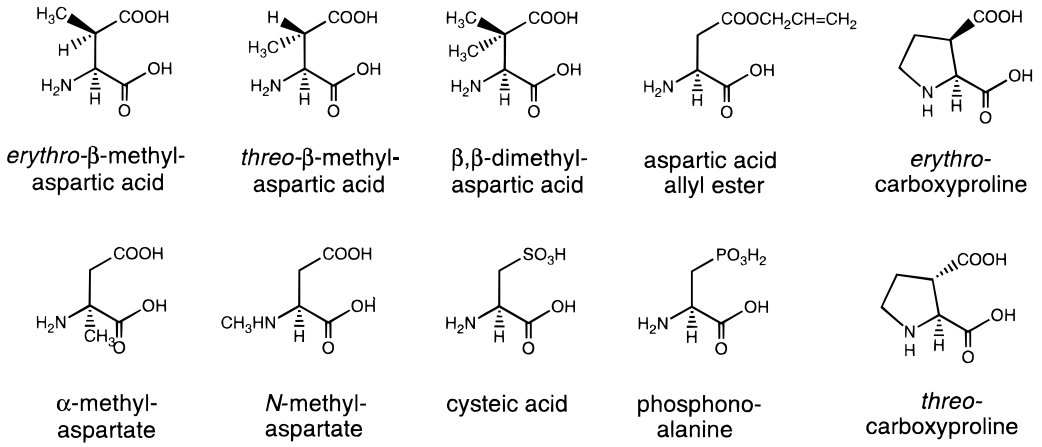

Figure 6. Structures of the modified amino acids incorporated into positions 10 and 27 of dihydrofolate reductase.

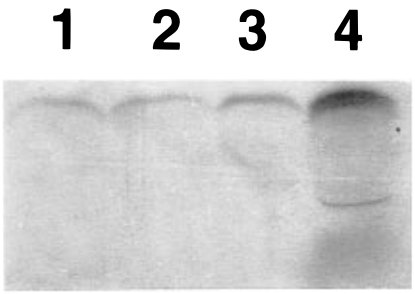

Figure 7. Autoradiogram of a 20% SDS-polyacrylamide gel showing crude and purified [^{35}S]methionine-labeled DHFRs synthesized using pTN2H4 mRNA (UAG at codon 10) and $tRNA_{CUA}$ activated with the following amino acids: lane 1, α-methylaspartic acid; lane 2, *N*-methylaspartic acid; and lanes 3 and 4, aspartic acid. Lanes 1−3 contained DHFR purified on Ni-NTA agarose prior to PAGE; lane 4 contained the crude product from the protein synthesis reaction.

ficiency than the charged aspartic acid analogues, consistent with earlier observations.[10r] Second, although some interexperiment variation in yields was noted, the efficiency of incorporation of any given amino acid into position 10 was not dramatically different than that noted for position 27. While not yet explored in detail, it may also be noted that protection of aspartic acid as its allyl ester substantially enhanced the efficiency of incorporation of a polar modified amino acid into protein. In spite of the technical limitations reflected in the present study, a substantial variety of amino acid analogues were, nonetheless, successfully incorporated into DHFR in position 10 or 27 (Table 1 and Figure 6).

Purification and Analysis of Modified DHFRs. Purification of the modified DHFRs was carried out in two steps. First, the elaborated proteins were purified by chromatography on DEAE Sepharose CL-6B, which permitted removal of hemoglobin present in the rabbit reticulocyte lysate. Subsequent application of the modified DHFRs to an Ni-NTA agarose column effected specific retention of those proteins having a hexahistidine moiety at the N-terminus; protein factors introduced with the rabbit reticulocyte lysate as well as truncated DHFRs (e.g., those formed by initiation at internal methionine codons, cf Figures 4 and 5) were thereby eliminated. The effectiveness of this procedure is readily apparent in Figure 7, which illustrates the purification of three different DHFRs.

That the modified amino acids shown in Figure 6 actually were incorporated at the intended sites was verified by degradation of several of the DHFRs modified at position 10. These DHFRs were elaborated in the presence of ^{35}S-methionine, purified, and then digested with Glu-C endoproteinase which hydrolyzes peptide bonds specifically at the carboxamide side of glutamic acid residues.[16] As shown in Scheme 2 and Figure 2, one of the ^{35}S-labeled products should be a 17-amino acid peptide encompassing amino acids 1-17 of DHFR.

Nonradiolabeled peptide standards of this 17-amino acid peptide containing valine, aspartic acid, or *threo*-*β*-methylaspartic acid at position 10 were prepared by chemical synthesis. Each of these peptides was added to a Glu-C endoproteinase digest of ^{35}S-labeled DHFR elaborated by *in vitro* protein biosynthesis and putatively containing the same amino acid at position 10. Each mixture was then fractionated by reverse phase HPLC with simultaneous detection of UV absorption and radioactivity. As is clear from Figure 8, each of the proteolytic digests contained a ^{35}S-labeled peptide that comigrated with the authentic synthetic standard. It may be noted that the relative retention times of these peptides accurately reflected the lipophilicity of the amino acid at position 10.

Properties of the Modified DHFRs. The modified DHFRs were assayed for their abilities to bind to the substrate analogue methotrexate[2] and for their catalytic competence in converting dihydrofolic acid to tetrahydrofolic acid. Methotrexate binding was assayed by applying solutions of the Ni-NTA agarose-purified DHFRs to an agarose column containing immobilized methotrexate. The column was washed with low and then high salt buffers and finally with a high salt buffer containing 10 mM folic acid. The elution of the modified DHFRs was monitored by scintillation counting of aliquots withdrawn from individual fractions.

Position 10, which contains valine in wild-type DHFR, was chosen for replacement because it is not known to be associated with any DHFR function; it was anticipated all DHFRs modified at this position would bind to methotrexate-agarose. As illustrated in Figure 9 and Table 2, this proved to be true for wild-type DHFR and also for DHFRs elaborated *in vitro* with valine, aspartic acid, and phenylalanine at position 10.

Position 27 normally contains aspartic acid; when employed at pH 7.0, none of the other 19 amino acids normally incorporated into proteins ribosomally can substantially support the catalytic function of DHFR.[2,3] As shown in Figure 10 and Table 2, the introduction of valine or phenylalanine into position 27 afforded DHFRs that would not bind to methotrexate-agarose. In comparison DHFRs produced *in vitro* containing aspartic acid, cysteic acid, and *erythro*-carboxyproline at position 27 all bound to the methotrexate affinity resin. It is interesting that for a few of the elaborated proteins capable of binding to methotrexate-agarose, there was also a peak of radiolabel that eluted in the high salt wash and which may correspond to protein that is not folded properly (Figure 10). To determine whether this putative protein might slowly equilibrate with folded protein for the DHFR analogues, we reapplied a few of the purified,

(16) Drapeau, G. R. *Methods Enzymol.* **1976**, *45*, 469.

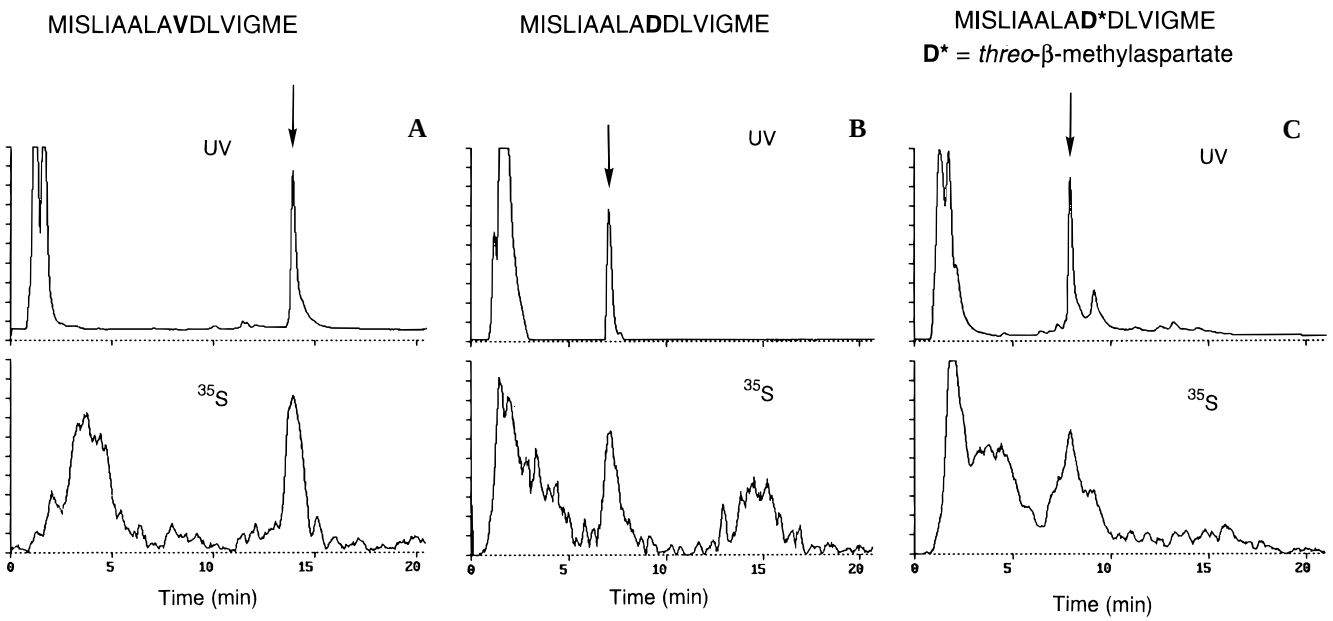

Figure 8. Comparative analysis by C^{18} reverse phase HPLC of [^{35}S]methionine-labeled 17-amino acid peptides derived by Glu-C endoproteinase treatment of modified DHFRs, vs authentic synthetic samples of the putative products: panel A, peptide containing valine at position 10; panel B, peptide containing aspartic acid at position 10; and panel C, peptide containing *threo*-β-methylaspartic acid at position 10.

Table 2. Binding of Structurally Modified DHFRs to a Methotrexate Affinity Column

	binding capability of DHFR altered at	
amino acid substitution	position 10	position 27
aspartic acid	+	+
erythro-carboxyproline		+
cysteic acid		+
phenylalanine	+	−
valine	+	−

Table 3. Relative Catalytic Efficiencies of Modified DHFRs

	relative efficiencies of DHFR modified at	
amino acid	position 10	postion 27
aspartic acid		1.0
β,β-dimethylaspartic acid		0.74
erythro-β-methylaspartic acid		0.86
threo-β-methylaspartic acid		0.76
phenylalanine	1.0	0
valine	1.0	0

folded proteins to another methotrexate column. As illustrated for DHFR containing cysteic acid in position 27, each of the purified proteins tested eluted from the second column only when treated with folic acid.

A number of the modified DHFRs were also prepared on a larger scale and tested for catalytic competence. It was shown that DHFR containing the hexahistidine fusion peptide at its N-terminus displayed the same characteristics as wild-type DHFR in converting dihydrofolic acid to tetrahydrofolic acid. Further, DHFR produced by *in vitro* translation and containing aspartic acid at position 27 had the same properties as naturally derived DHFR[cf. ref 4c]. In comparison, incorporation of valine or phenylalanine at position 27 rendered DHFR dysfunctional, as expected.[2,3]

Of particular interest were the DHFRs containing *erythro*- and *threo*-β-methylaspartic acids and β,β-dimethylaspartic acid, all of which reproducibly converted dihydrofolate to tetrahydrofolate about 74−86% as well as wild-type DHFR in multiple turnover experiments (Table 3). In order to determine whether this was due to an intrinsic alteration in the rate of hydride transfer from NADPH or to limitation of some other step in the overall process of tetrahydrofolate production (e.g., product release from DHFR), we studied the facility of product formation in the presence of NADPH vs NADPD at two different pH values.[17]

Table 4. Tetrahydrofolate Production by a Modified DHFR Containing β,β-Dimethylaspartate at Position 27

DHFR	pH	relative product formation (NADPH/NADPD)
aspartic acid	7.0	1.0 ± 0.1
aspartic acid	8.1	1.6 ± 0.1
β,β-dimethyaspartic acid	7.0	1.9 ± 0.6

For the wild-type DHFR at 37 °C, we found that

$$\frac{V_{\text{pH 7.0}}}{V_{\text{pH 8.1}}} = 1.4 \pm 0.1$$

in good qualitative agreement with previous results obtained under somewhat different assay conditions.[17] The DHFR containing β,β-dimethylaspartic acid at position 27 gave a maximum velocity only slightly less than observed for wild-type DHFR at pH 7.0 (cf. Table 3). In contrast, at pH 8.1 this modified DHFR was much less efficient, such that it was difficult to obtain a precise value for the maximum velocity. We estimate that

$$\frac{V_{\text{pH 7.0}}}{V_{\text{pH 8.1}}} > 3$$

for the modified DHFR.

Measurement of the maximum velocity of the wild-type and modified DHFRs was also carried out in the presence of NADPD. As shown in Table 4, at pH 7.0 the maximum velocity of product formation for the wild-type enzyme was the same within experimental error in the presence of NADPH or NADPD. At pH 8.1, the maximum velocity of product formation was 1.6 times greater in the presence of NADPH than NADPD, reflecting the known diminution of the rate of hydride transfer at higher pH.[17] For the DHFR containing β,β-

(17) (a) Fierke, C. A.; Johnson, K. A.; Benkovic, S. J. *Biochemistry* **1987**, *26*, 4085. (b) Chen, J.-T.; Taira, K.; Tu, C.-P. D.; Benkovic, S. J. *Biochemistry* **1987**, *26*, 4093.

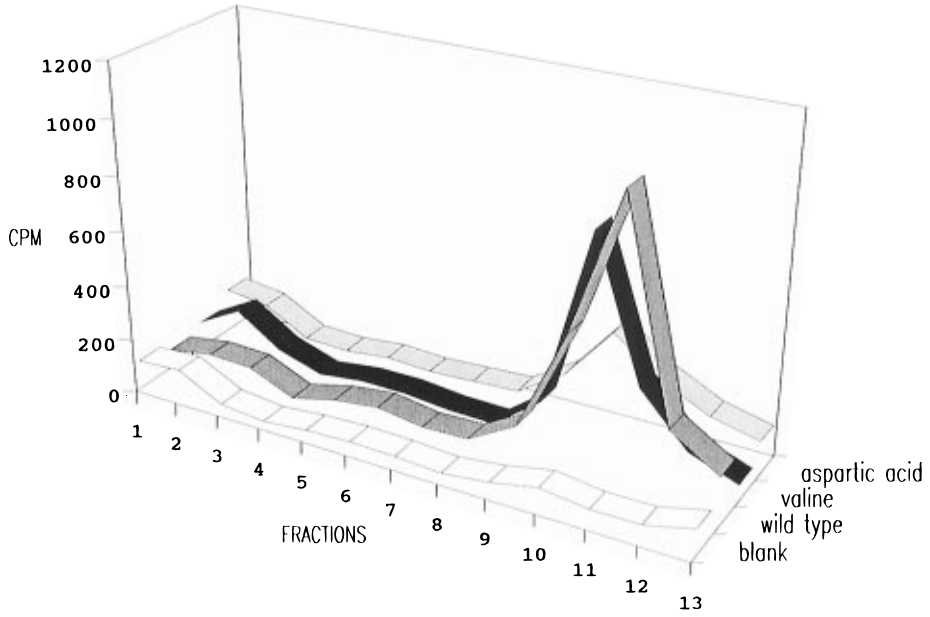

Figure 9. Elution of DHFRs from a methotrexate-agarose affinity column. DHFRs synthesized from pTZN2H4 mRNA with valine and aspartic acid at position 10 were compared with wild-type DHFR elaborated using pTHis 15 mRNA. The column was washed with potassium phosphate buffer, pH 6 (fractions 2−4), phosphate buffer containing 1 M KCl (fractions 5−8), and then phosphate buffer containing 1 M KCl and 10 mM folic acid (fractions 9−13).

dimethylaspartic acid at position 27, the maximum velocity of product formation was found to be 1.9 times greater in the presence of NADPH than NADPD even at pH 7.0, suggesting strongly that hydride transfer was the rate-limiting step from the modified enzyme even at pH 7.0. This was consistent with the finding that product formation by the modified enzyme was difficult to measure at pH 8.1 in the presence of NADPH (*vide supra*) and virtually undetectable in the presence of NADPD.

Discussion

The results presented clearly demonstrate that a number of modified amino acids have been incorporated into positions 10 and 27 of dihydrofolate reductase. The modified proteins had the same electrophoretic mobility on SDS-polyacrylamide gels as wild-type DHFR; the mobility of the latter relative to a set molecular weight markers was consistent with the calculated M_r of 19 196 expected for *E. coli* DHFR containing a 9-amino acid fusion peptide at the N-terminus (Figures 2 and 3). More definitively, proteolysis of several of the DHFRs modified at position 10 with Glu-C endoproteinase afforded peptides identical with authentic synthetic standards as judged by HPLC analysis (Figure 8).

In vitro synthesis of the modified DHFRs, which involved suppression of a nonsense codon in each case, was carried out successfully using a commercially available rabbit reticulocyte lysate system. This system was employed previously for the overexpression of DHFR according to a strategy that coupled the polymerase chain reaction with transcription and translation; the derived protein was fully competent as a catalyst relative to wild-type DHFR isolated from *E. coli*.[5]

Because the elaboration of proteins in cell free systems requires the addition of protein factors in greater total abundance than the protein species actually synthesized, it is essential to have some strategy for purification of the derived proteins. In the present case, a hexahistidine moiety included as a fusion peptide at the N-terminus was able to bind to a Ni-NTA agarose affinity resin; subsequent elution with a high salt buffer containing 10 mM imidazole effected elution of the purified protein. In principle, it would have been possible to effect purification of the elaborated DHFRs on the methotrexate-agarose columns used to analyze the binding capabilities of the proteins (Figures 9 and 10). However, this procedure would not have been applicable to modified DHFRs such as the ones containing phenylalanine and valine at position 27, which were formed in good yields (Table 1) but were incapable of binding to the methotrexate affinity resin.

One interesting facet of the synthesis of DHFR-containing modifications at position 10 was the appearance of a band slightly shorter than the full length product even when no suppressor tRNA was present (Figure 4). This byproduct was readily separated from the full length product because it was not retained on the Ni-NTA agarose column during purification; the observed behavior on Ni-NTA agarose indicates that this shorter protein must lack the N-terminal fusion peptide. However, this product was not separated from the full length product on a methotrexate agarose affinity column, indicating that the missing amino acids do not extend into the active site. It seems likely that this shorter protein results from initiation of translation from the ATG codon for Met-16 (Figure 2). One more product located on the gel between the full length product and the protein discussed above seems likely to have been produced from the same mRNA by "context-dependent leaky scanning" [18] from the ATG codon for Met-1. It also failed to bind to Ni-NTA agarose.

The suppression efficiencies obtained depended very substantially on the nature of the amino acid incorporated. The aspartic acid derivatives studied, which are of the greatest interest in the present case from the perspective of probing enzyme mechanism, were incorporated much less efficiently than valine and phenylalanine from the same suppressor tRNA. Other hydrophobic amino acids tested in the same system were also incorporated with greater facility than aspartic acid (analogues). The lower level of incorporation of charged amino acids has been noted previously[10r] and may well represent a general property of such amino acids. At present it is less clear whether charged amino acids participate in peptide bond formation with lesser facility in response to all codons that specify such aminoacyl-tRNAs or whether the need for suppressor tRNAs to compete with release factors that also interact

(18) Kozak, M. *J. Cell Biol.* **1990**, *115*, 887.

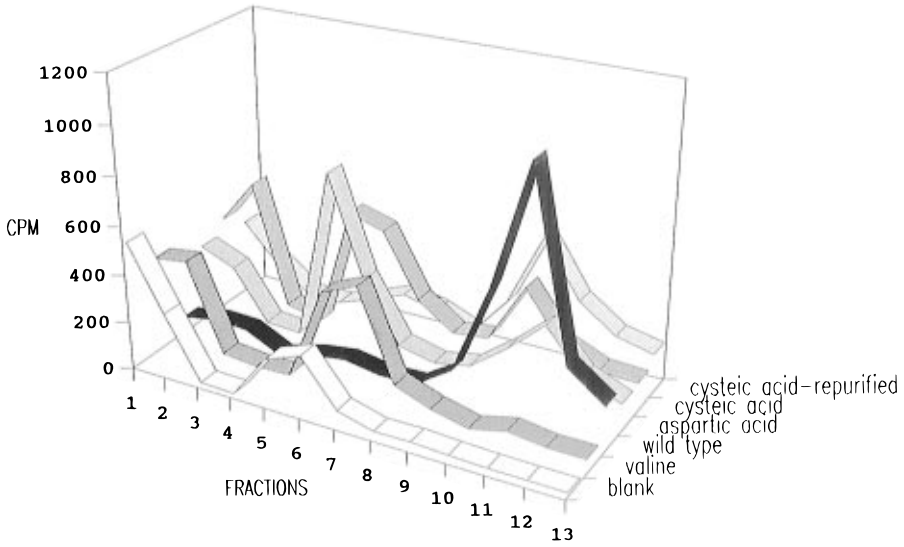

Figure 10. Assay of DHFRs altered at position 27 on a methotrexate-agarose affinity column. The binding assay was run as indicated in the legend to Figure 9.

with nonsense codons[19] places those species bearing charged amino acids at some selective disadvantage.

From a practical perspective, the observation that the allyl ester of aspartic acid could be incorporated with much greater efficiency than aspartic acid itself (Table 1) offers great promise for the elaboration of modified proteins containing polar, modified amino acids in enhanced yields. While obvious technical issues such as the optimal choice of protecting groups, their introduction into aminoacylated dinucleotides, and the reliable removal of the protecting groups from the elaborated proteins (many of which will have folded around the protecting group) still require resolution, these are being addressed at present in the belief that a much greater variety of modified amino acids can be introduced with facility in this fashion.

In order to demonstrate the potential of the present technique for dissecting enzyme mechanism at higher precision than can be achieved using site directed mutagenesis with the 20 amino acids that nature uses to construct proteins, we studied three of the modified DHFRs. As shown in Table 3, substitution of the aspartic acid at position 27 with aspartic acid analogues bearing one or two methyl groups at the β-position of the side chain resulted in a modest but reproducible diminution in the maximum velocity of tetrahydrofolate production. In multiple turnover experiments of this type, the rate limiting step for tetrahydrofolate production has been shown to involve release of the formed product rather than hydride transfer per se. To determine whether the diminution in tetrahydrofolate production observed for the modified DHFRs was due to an alteration of active site geometry, which further diminished the rate of product release, or to a change in the rate limiting step, we measured the maximum velocity of product formation in the presence of NADPH vs NADPD.[17] As shown in Table 4, the wild-type enzyme exhibited an isotope effect only at higher pH, reflecting a change in the rate limiting step to hydride transfer, as noted previously.[17] Interestingly, the DHFR bearing β,β-dimethylaspartic acid at position 27 exhibited a significant isotope effect even at pH 7.0, arguing that the introduction of the two methyl groups within the active site at position 27 altered the rate limiting step for tetrahydrofolate production.

It is interesting that the simple introduction of one or two methyl groups within an active site residue in orientations that would seem from the X-ray crystal structure of the $NADP^+$–dihydrofolate–DHFR compex[3f] to be potentially tolerant of substitution nonetheless altered the production of tetrahydrofolate by DHFR both quantitatively and qualitatively. Assuming that the structurally modified DHFRs bind substrate in the same fashion as the wild-type enzyme, the alterations associated with introduction of the methyl group in *erythro*-β-methylaspartate may result from steric interaction with the carbonyl of Leu24, while the methyl group in the *threo* isomer is relatively close to the side chain of Phe153. Obviously, it is also possible that the introduction of these methyl groups has resulted in a more substantial reorganization of the enzyme active site. In any case, these alterations in the behavior of the enzyme demonstrate that small, defined structural changes in enzyme structure have the wherewithal to produce functional changes that can facilitate a better understanding of the relationship between enzyme structure and function.

We are presently utilizing misacylated tRNAs to modify DHFR at multiple positions in order to carry out the dissection of enzyme function at high resolution.

Experimental Section

General Methods. Nuclease-treated rabbit reticulocyte lysate, DNA polymerase I (Klenow fragment), T4 DNA ligase, and restriction endonucleases *Kpn*I, *Hin*dIII, *Bam* HI, and *Bcl*I were purchased from Promega Corp. Restriction endonucleases *Pfl*MI and *Fok*I as well as purified acylated bovine serum albumin (BSA) were from New England Biolabs. Methotrexate immobilized on agarose (MTX agarose), DEAE Sepharose CL-6B, β-NADPH, folic acid, dihydrofolic acid, and Glu-C endoproteinase were obtained from Sigma Chemicals. Kits for plasmid isolation and for purification of proteins on Ni-NTA agarose were purchased from QIAGEN Inc. (Chatsworth, CA). Synthetic oligonucleotides were obtained from Cruachem, Inc. (Dulles, VA) or Midland (Midland, TX). *Escherichia coli* competent cells were purchased from Stratagene Cloning Systems (LaJolla, CA). AmpliScribe transcription kits were obtained from Epicentre Technologies (Madison, WI); [^{35}S] methionine (1000 Ci/mmol) was from Amersham Corporation.

Ultraviolet spectral measurements were made using Perkin-Elmer Lambda Array 3840 or Hewlett Packard 8451A Diode Array Spectrophotometers equipped with a thermal control unit. Radioactivity measurements were made with a Beckman LS-100 C liquid scintillation counter. Phosphorimager analysis was performed using a Molecular Dynamics 300 E PhosphorImager equipped with ImageQuant software. HPLC analysis was carried out using a Varian Model 2050 system; [^{35}S]-containing material was monitored by a β-RAM radioactivity detector (Insus Systems, Inc., Tampa, FL). Peptide mass spectral analysis was carried out on a Finnigan-MAT Lasermat MALDI-TOF mass spectrometer.

Construction of Expression Plasmids Carrying the Gene for Dihydrofolate Reductase. Plasmid pTZRKE, a derivative of pTZ19R

(19) Caskey, C. T. *Trends Biochem. Sci.* **1980**, *5*, 234.

encoding wild-type DHFR[6] under the control of a T7 promoter, was modified for this study. The *Bcl*I-*Pfl*MI fragment from pTZRKE coding for the wild-type DHFR amino acid sequence from Met-1 to Ala-19 (Figure 2) was replaced by a synthetic oligonucleotide duplex containing the same sequence except for the replacement of the GTA codon for Val-10 by a TAG codon. The resulting plasmid, pTZN2, was linearized with *Bcl*I and a synthetic duplex coding for nine amino acids that included hexahistidine was ligated to the linearized plasmid DNA, affording plasmid pTZN2H4 (Figures 1 and 2). The same oligonucleotide duplex encoding hexahistidine was incorporated into the *Bcl*I site of pTZRKE and PTZ27R, the latter of which was identical to pTZRKE except for the replacement of the GAT codon for Asp-27 by TAG. The resulting plasmids were denoted pTHis15 and pTZ27R2H11, respectively. The nucleotide sequences in all of the plasmids were verified by restriction analysis and Maxam−Gilbert DNA sequencing.[20]

Construction of an Expression Plasmid for the *in Vitro* Transcription of Yeast $tRNA^{Phe}_{CUA}$ (-CA). A DNA duplex containing the T7 promoter, the nucleotide sequence corresponding to yeast $tRNA^{Phe}_{CUA}$ (-CA)[10d,12] and appropriately placed *Kpn*I, *Hin*dIII, *Bst*NI, and *Fok*I cleavage sites, was constructed using two overlapping synthetic oligonucleotides having the sequences pCCAACTGGTACCGCTGCAGTAATACGACTCACTATAGCGGATTTAGCTCAGTTGGGAGAGCGCCAGACTCTAAATCTG and pTTTACTAAGCTTGGATGGATCACCTGGTGCGAATTCTGTGGATCGAACACAGGACCTCCAGATTTAGAGTCTGGC. The oligonucleotides were purified on 8% denaturing polyacrylamide gels, then 5 μg of each was annealed in 100 μL of 10 mM Tris HCl, pH 7.5, containing 5 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, and 50 μg/mL of acylated BSA. The solution was heated to 70 °C and then cooled slowly over a period of 30 min. Polymerization was carried out in the presence of 1 mM NTPs and 5 units of DNA polymerase I (Klenow fragment) at 37 °C for 30 min. The double-stranded DNA was purified on a 6% nondenaturing polyacrylamide gel, digested with restriction endonucleases *Kpn*I and *Hin*dIII, and then ligated to pUC19 DNA that had been digested with the same restriction enzymes. The nucleotide sequence of the resulting plasmid was verified by restriction analysis and dideoxy sequencing.[21]

Construction of an Expression Plasmid for the *in Vitro* Transcription of *E. coli* $tRNA^{Ala}_{CUA}$ (-CA). A plasmid (pAla35) containing a gene for *E. coli* $tRNA^{Ala}_{CUA}$ with a U → C mutation at position 70[13] was obtained from Dr. Paul Schimmel. The gene, flanked by an upstream T7 promoter, was incorporated into commercially available plasmid pSP65 between *Eco*RI and *Bam*HI sites. A *Fok*I site was incorporated into the resulting plasmid by replacement of a short fragment excised by treatment with *Bam*HI and *Pst*I endonucleases with the duplex formed from the synthetic oligonucleotides pGATCCGCATCCTTCTGCA and pGAAGGATGCG. The nucleotide sequence of the derived plasmid was confirmed by restriction analysis and dideoxy sequencing.[21]

***In Vitro* Transcription of $tRNA_{CUA}$s.** The plasmid DNAs were linearized using *Fok*I and then transcribed using an AmpliScribe T7 transcription kit in a buffered reaction mixture (500 μL total volume) containing 7.5 mM of each of the four NTPs, 10 mM dithiothreitol, 20 nM template DNA, and 1 μL/10 μL of T7 RNA polymerase preparation. The transcribed tRNA was recovered from the reaction mixture by precipitation with 2.5 volumes of ethanol, then collected by centrifugation, and dried under vacuum. The crude tRNA was then dissolved in 80% formamide containing 0.02% xylene cyanole FF and bromophenol blue, applied to an 8% denaturing polyacrylamide gel (40 cm × 20 cm × 2 mm), and subjected to electrophoresis at 800 V for 3 h. The RNA bands were visualized by UV shadowing,[22] excised from the gel and recovered by the crush and soak method[20] with 100 mM Na acetate, pH 4.6, containing 1 mM EDTA and 0.01% SDS at 4 °C for 12 h. The tRNA was recovered by ethanol precipitation, dried, and then redissolved in RNase-free water stored in aliquots at −80 °C.

Synthesis of Misacylated tRNAs. Ligation reactions were carried out in 50 μL (total volume) of 50 mM Na Hepes buffer, pH 7.5, containing 5 μL (∼0.2 nmol) of $tRNA_{(CUA)}$(-CA), 0.5 A_{260} unit (∼20 nmol) of an aminoacyl-pdCpA, 0.5 mM ATP, 15 mM $MgCl_2$, 10% dimethyl sulfoxide and 100 units of T4 RNA ligase. Reaction mixtures were incubated at 37 °C for 25 min and then quenched by the addition of 5 μL of 3 M Na acetate, pH 4.5. The (aminoacylated) tRNA was precipitated with 2.5 volumes of ethanol, collected by centrifugation, washed with 70% ethanol, and dried. The product was redissolved in 1 mM KOAc to a final concentration of 1 μg/μL and then irradiated with a 500 W mercury-xenon lamp using Pyrex and water filters. The protected aminoacyl-tRNA was cooled in an ice bath during irradiation, which was typically carried out for 2 min for amino acid derivatives containing one NVOC group and 5 min for derivatives with two NVOC groups. The deprotected aminoacyl-tRNAs were used in *in vitro* suppression experiments immediately following deprotection.

The extent of ligation to afford misacylated tRNAs could be analyzed by electrophoresis on a (200 × 200 × 0.8 mm) 8% polyacrylamide gel containing 7 M urea in 90 mM Tris-borate buffer, pH 8.2, at 800 V for 3 h. The deblocking conditions were optimized using protected aminoacyl-pdCpA's; product analysis was carried out by HPLC.

Synthesis of mRNA by *in Vitro* Transcription. The plasmid DNAs were linearized with *Bam*HI and then transcribed using an AmpliScribe T7 transcription kit as described above for *in vitro* $tRNA_{CUA}$ transcription. The transcribed mRNA solution was extracted successively with phenol and chloroform. The mRNA was precipitated from solution with 2.5 volumes of ethanol, washed with 70% ethanol, and dried. The mRNA was dissolved in RNase-free water and stored in aliquots at −80 °C.

***In Vitro* Synthesis of Dihydrofolate Reductase.** In a typical experiment, DHFR was synthesized in a reaction mixture (25−300 μL total volume) that contained *per 100* μL: 70 μL of methionine-depleted, nuclease-treated rabbit reticulocyte lysate, 80 μCi of [^{35}S]-L-methionine (1000 Ci/mmol), 2 μL of a solution 1 mM in 19 amino acids used in ribosomal protein synthesis (but lacking methionine), 8 μg of the appropriate mRNA, and 10 μg (∼0.4 nmol) of deprotected misacylated $tRNA_{CUA}$. The reaction mixture was incubated at 30 °C for 2 h. *In vitro* translation of control pTHis15 mRNA was carried out without added misacylated $tRNA_{CUA}$. Aliquots (typically 1 μL) were utilized for analysis by 20% SDS-PAGE.[23] Autoradiography of the gels was carried out to determine the location of ^{35}S-labeled protein; quantification of the bands was carried out using a phosphorimager.

For the synthesis of DHFR for enzymatic assay, the reaction mixtures contained an additional 2 μL (per 100-μL of incubation solution) of 1 mM amino acids expect leucine, which was included as [^{3}H]leucine for purposes of protein quantification.

Purification of Synthesized DHFRs. *In vitro* translation mixtures (300 μL) containing ^{35}S-labeled protein were applied to a 600-μL DEAE Sepharose CL-6B column that had been equilibiated with 5 mM K phosphate, pH 7.0. The column was washed with five 100-μL portions of 5 mM K phosphate buffer, pH 7.0; ^{35}S-labeled protein was then washed from the column with five 100-μL portions of 5 mM K phosphate, pH 7.0, containing 1 M KCl.

The fractions containing the radiolabeled proteins of interest were applied to a 200-μL Ni-NTA agarose column that had been equilibrated with 50 mM Na phosphate, pH 8.0, containing 300 mM NaCl, 20 mM imidazole, and 100 μg/mL BSA. The column was washed with five 100-μL portions of the same buffer; the protein was then eluted from the column by washing with five 100-μL portions of 50 mM Na phosphate, pH 8.0, containing 300 mM NaCl, 250 mM imidazole, and 10 μM BSA. The amount of ^{35}S-labeled protein in each fraction was determined by liquid scintillation counting of a portion of each.

Binding of DHFRs to a Methotrexate Affinity Resin. A column containing fifty μL of MTX agarose was equilibrated with 400 μL of 10 mM K phosphate, pH 6.0, containing 10 μM BSA. Aliquots (10−50 μL) of the DHFR purified on Ni-NTA agarose were combined with 50 μL of 10 mM K phosphate, pH 6.0, containing 100 μg/mL BSA and 50 μL of the equilibrated MTX agarose resin; the mixture was maintained at 25 °C for 1 h. The resin was then washed successively with three 100-μL aliquots of the same buffer and four 100-μL aliquots of 200 mM K phosphate, pH 6.0, containing 1 M KCl and 10 μM BSA. The resin was then combined with 10 μL of 200 mM K phosphate, pH 9.0, containing 1 M KCl, 10 mM folic acid, and 10 μM BSA, and the mixture was maintained at 25 °C for 1 h. The resin was then washed with four 100-μL portions of 200 mM K phosphate,

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(21) Sanger, F.; Nicklen, S.; Coulson, A. R. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 5463.

(22) Hassur, S. M.; Whitlock, H. W. *Anal. Biochem.* **1974**, *59*, 162.

(23) Laemmli, U. K. *Nature* **1970**, *227*, 680.

pH 9.0, containing 1 M KCl, 10 mM folic acid, and 10 μM BSA. Aliquots (10−50 μL) of each fraction were used for determination of radioactivity.

Quantitation of DHFR Activity. The enzymatic activity of DHFR was determined by oxidation of NADPH, the latter of which was monitored by the decrease in absorption at 339 nm according to the method of Baccanari et al.[24] Following purification on DEAE Sepharose CL-6B and Ni-NTA agarose, 20−100 μL of the DHFR fraction was diluted to 100 μL as neccesary with 50 mM sodium phosphate, pH 8.0, containing 300 mM NaCl, 250 mM imidazole, and 100 μg/mL bovine serum albumin. The (diluted) aliquots were included in assays (1 mL total volume) containing 100 mM imidazole, pH 7.0, 10 mM β-mercaptoethanol, 100 μM dihydrofolic acid, and 100 μM NADPH. The reaction was carried out for 10−30 min at 37 °C. Enzymatic activity was determined by spectrophotometric determination of NADPH consumed, by monitoring at 339 nm. The amount of protein used for assay was determined from the amount of ^{35}S-labeled protein that comigrated with DHFR on SDS polyacrylamide gels. NADPH consumption was corrected for background obtained when mRNA translation was carried out in the absence of misacylated suppressor tRNA.

For activity measurement at variable pH, Tris-HCl was employed as the buffer.

For measurement of DHFR activity in the presence of NADPD, deuterated (4*R*)-[^{2}H]NADPH was prepared by the procedure of Jeong and Gready.[25] Purification was carried out by reverse phase hplc on a 250 × 10 mm Econosil C_{18} column (Alltech); elution was effected with 50 mM $(NH_4)_2CO_3$ solution at a flow rate of 2 mL/min and detected at 260 and 339 nm. Fractions having a A_{260}/A_{339} ratio of ∼2.3 were combined and isolated by lyophilization.

Syntheses of Authentic 17-Amino Acid Peptides. The peptides were synthesized using a RaMPS peptide synthesizer (Dupont) which employed Fmoc-Glu(O-*t*-Bu)-Wang resin.[26] Fmoc esters activated *in situ* in DMF in the presence of HOBT were coupled to the peptide resin; coupling steps were monitored by the Kaiser test.[27] The synthesized peptides were cleaved from the resin with 94:5:1 trifluoroacetic acid−thioanisole−ethanedithol at 25 °C over a period of 18 h. The solution containing the cleaved product was lyophilized, affording a white powder. The residue was dissolved in 20% aqueous sodium dodecyl sulfate with sonication and purified by reverse phase HPLC on a C_{18} column (Alltech, 250 × 10 mm). The column was washed with a linear gradient from 30 → 60% acetonitrile in water containing 0.1% trifluoroacetic acid; the flow rate was 1.0 mL/min over a period of 30 min. After lyophilization, the purified peptides were obtained as fluffy white solids. The peptides were analyzed by mass spectrometry (MALDI TOF): MISLIAALA**V**DLVIGME, *m/z* 1805.6 (calculated 1802.4); MISLIAALA**D**DLVIGME, *m/z* 1820.5 (calculated 1818.3); MISLIAALA***β*MeD**DLVIGME, *m/z* 1831.3 (calculated 1832.3).

Analysis of DHFRs Containing Alterations at Position 10. DHFRs elaborated in the presence of ^{35}S-methionine and putatively containing valine, aspartic acid, or *threo*-β-methylaspartic acid at position 10 were purified successively on DEAE Sepharose CL-6B and then Ni-NTA agarose columns, as described above. The purified proteins were dialyzed against water for 1 h and then digested with 4 μg of Glu-C endoproteinase in reaction mixtures (60 μL total volume) that contained 50 mM $NaHCO_3$, pH 7.8, and 2 mM EDTA. The reaction mixtures were incubated overnight at 37 °C. An aliquot (10−30 μL) of each reaction mixture was treated with ∼1 mg of authentic heptadecapeptide dissolved in 100 μL of 20% SDS. The mixtures were analyzed by reverse phase HPLC on an Econosphere C_{18} column (Alltech, 100 × 4.6 mm) using a linear gradient of 25% acetonitrile in 100 mM aqueous Na_2SO_4 → 50% acetonitrile in 100 mM aqueous Na_2SO_4 at a flow rate of 1.0 mL/min over a period of 20 min. The elution of ^{35}S-labeled material (β-RAM radioactivity detector) and UV-absorbing material (215 nm) were monitored simultaneously.

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