THYMIDYLATE SYNTHETASE: STUDIES ON THE PEPTIDE CONTAINING COVALENTLY BOUND 5-FLUORO-2'-DEOXYURIDYLATE AND 5,10-METHYLENETETRAHYDROFOLATE

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SUMMARY: Studies are reported on the FdUMP-CH_2-H_4folate-peptide obtained upon proteolysis of the complex formed from thymidylate synthetase, FdUMP and 5,10-CH_2-H_4folate. Contrary to a previous report from this laboratory, the peptide does contain a cysteine residue. The sequence of the largest peptide obtained is Ala-Leu-Pro-Pro-(His,Cys)-Thr. Quantitative modification of the histidine residue with the Pauly reagent indicates that imidazole is not directly linked to the nucleotide. The stability of the peptide indicates the covalent bond to the cofactor involves its 5-nitrogen; from this, it may be concluded that the reactive form of the cofactor is the 5-iminium ion.

Thymidylate synthetase reacts with 5-fluoro-2'-deoxyuridylate (FdUMP) and 5,10-methylenetetrahydrofolate (CH_2-H_4folate) to form a ternary complex in which all components are covalently attached (1-4). Since the complex formed is analogous to a steady-state intermediate of the normal enzymic reaction(5), much effort has been expended in attempts to elucidate its structure and mechanism of formation. All available evidence indicates that a nucleophile of the enzyme adds to the 6-position of FdUMP, and the 5-position of the pyrimidine is coupled to H_4folate *via* the methylene group of the cofactor. We have recently reported (6) that proteolysis of the complex yields a peptide which is covalently attached to FdUMP and CH_2-H_4folate in the same manner.

We have continued investigations of this peptide with the hope that complete knowledge of its structure will yield pertinent information regarding the mechanism of thymidylate synthetase and its interaction with FdUMP. In this report we describe (*i*) the finding of Cys in the peptide, (*ii*) sequence studies of the peptide, (*iii*) evidence that His is not the nucleophile attached to FdUMP, and (*iv*) evidence that the covalent linkage to H_4folate is at the 5-nitrogen; the latter implicates the 5-iminium ion of the cofactor as the reactive electrophilic species. We note that the finding of Cys in the peptide represents a retraction of a result reported earlier from this laboratory (6).

MATERIALS AND METHODS: Thymidylate synthetase was obtained from an Amethopterin resistant strain of *Lactobacillus casei* (7) and was purified by previously described methods (3). Pronase was obtained from Calbiochem. Carboxypeptidase C and aminopeptidase M were from Rohm, and leucine aminopeptidase was from Worthington. Other materials have been previously described (3,8).

which differed in their sensitivity to several inhibitors of sporulation, viz.,APA, FAA, EP etc.(1,2,6). Polyacrylamide gel electrophoresis of cell free extracts of sporulating cultures of Bacillus cereus T demonstrated two aconitase bands;one of them showing activity predominantly at 5 hr and the other at 12 hr culture age (7).

In our previous communication (8), we had reported the partial purification and stability characteristics of early and late aconitase present in Bacillus cereus T. Since both the enzyme preparations could not be fractionated to a considerably high degree of purity, the fact that both of them got eluted from DEAE-cellulose column at different elution volumes may merely be an experimental artefact.To eliminate this possibility, the two enzyme activities obtained from the same sporulating culture were specifically separated by subjecting them, both individually as well as in mixture, to DEAE-cellulose column chromatography, polyacrylamide gel electrophoresis and Sephadex G-100 gel column chromatography.

MATERIALS AND METHODS

All investigations were made on Bacillus cereus T, obtained from United States Department of Agriculture, Washington, D.C., and subsequently maintained at this laboratory on nutrient agar slants. The organism was grown in the "glucose-yeast extract-minerals medium"(modified G medium)(9,10)by the "active culture technique"(11,12) at 30 ± 1 C on a gyrotary shaker (speed 160 rpm). All other cultural conditions and methods for preparation of cell free extracts, partial purification of early and late aconitase by ammonium sulphate fractionation, assay of aconitase activity and protein content have been reported previously (8,13). For specific separation of early and late aconitase, 5 and 12 hr aged cells were obtained from the same experiment and both the enzymes from their respective 45 - 80 per cent ammonium sulphate fractions were used for column chromatography on DEAE-cellulose.

DEAE-cellulose Column Chromatography: The resin was regenerated, packed upto a height of 20 cm in a glass column (1.5 x 30 cm), and equilibriated with "elution buffer" (phosphate buffer, 0.01M, pH 7.0) using standard procedures (13). Three identical columns of DEAE-cellulose were prepared in the usual manner. 8 ml samples each of early and late aconitase preparation (containing 5 and 10 mg protein, respectively) were loaded on two of the three columns separately. To the third column, a mixture of the 4 ml each of early and late aconitase preparation (containing 2.5 and 5 mg protein, respectively) was loaded. All the three columns were then run, one after other, in the same way and under identical conditions. 3 ml fractions were collected at 8 to 10 C and analysed for aconitase activity and protein content. The peak fractions were used for polyacrylamide gel electrophoresis and Sephadex G-100 gel column chromatography.

Table I. Amino Acid Compositions of FdUMP-CH_2-H_4folate-peptides[a]

Amino Acids	Peptide			Average Value
	P_1	P_2	P_3	
Ala	1.02	.32	0	
Leu	1.00	1.00	1.00	1.00
Pro	1.85	1.86	1.97	1.89
His	1.41	0.82	1.0	1.08
Half-cystine	---[b]	---[b]	---[b]	
Cys(O_3H)	---[c]	1.13	0.75	0.94
Thr	1.06	0.96	1.15	1.06
Glu	1.19	1.16	1.0	1.12

[a]Calculated on the basis of 1.00 residue of Leu.

[b]Analyzed as half-cystine; not detected. [c]Not determined.

Table II. Sequence of Peptide P_4

Edman Step	Peptide Sequence	Dansyl NH_2-terminal
0	Leu-(2Pro,His,Cys,Thr)	Dns-Leu
1	Leu-Pro-(Pro,His,Cys,Thr)	Dns-Pro
2	Leu-Pro-Pro-(His,Cys,Thr)	Dns-Pro
3	Leu-Pro-Pro-(His,Cys,Thr)	?
4	Leu-Pro-Pro-(His,Cys,Thr)	?
5	Leu-Pro-Pro-(His,Cys)-Thr	Dns-Thr

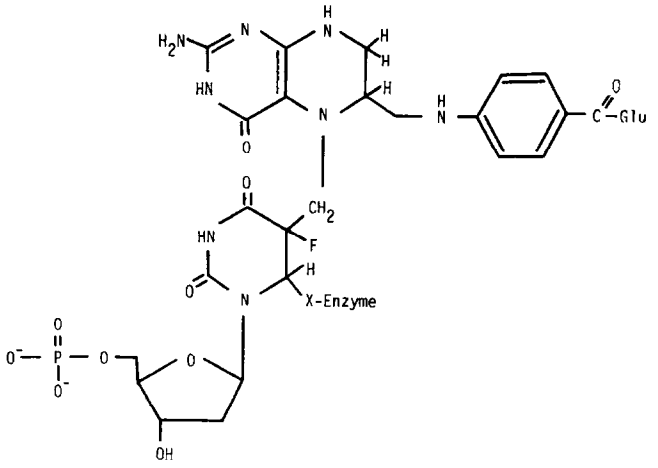

Figure 1. Structure depicting the FdUMP-CH_2-H_4folate-thymidylate synthetase complex and peptides derived upon its proteolysis.

amino acids could be definitively identified in the third or fourth cycle, but the fifth gave Dns-Thr. From the above data, the sequence of P_1 is Ala-Leu-Pro-Pro-X_1-X_2-Thr. From amino acid analysis, the fifth and sixth amino acids must be His and Cys, although their relative positions cannot be assigned.

The pronase derived [^{3}H]FdUMP-CH_2-H_4folate-peptide, P_1 (9.9 nmol), was reacted with the Pauly reagent for imidazoles (16,17) and shown to contain 10.6 nmol His or 1.07 His/peptide. The modified peptide was applied to a Sephadex G-25 column (1.1 x 28 cm) and eluted with 10 mM NaCl, pH 7.0; peptide P_1 and FdUMP are well retained on this column (6). A portion (3 ml) of the void volume was lyophilized and dissolved in 1.0 ml water. This contained 8700 dpm of tritium (3.5 nmol of FdUMP-peptide), had λ_{max} = 423, characteristic of the monoazoimidazole derivative, and A_{423} = 0/092; using ε_{423} = 23,600 (17) this corresponds to 3.9 nmol (1.1 His/peptide) and demonstrates that the imidazole of P_1 had been modified without disruption of the covalent linkage to FdUMP.

Peptide P_3, labeled with [2,6-^{14}C]FdUMP and [6-^{3}H]H_4folate had $^3H/^{14}C$ = 10.0 when first prepared. After storage at 0° for over six months in water, without precautions to avoid oxygen, a portion was rechromatographed on TEAE-cellulose (6). The sample was quantitatively recovered with an identical elution profile as when originally prepared and had $^3H/^{14}C$ = 10.0.

DISCUSSION: The general structure of the FdUMP-CH_2-H_4folate-thymidylate synthetase complex and FdUMP-CH_2-H_4folate-peptide is depicted in Figure 1. Some workers have taken the position that the sulfhydryl group of a Cys residue of

the enzyme is the nucleophile which attacks the 6-position of dUMP in the normal enzymic reaction, and FdUMP in the formation of complexes (4,18,19). Considering the high nucleophilicity of thiols and the fact that sulfhydryl groups of the enzyme are necessary for activity (20), it is intuitively reasonable to consider Cys as a prime candidate for this role.

Nevertheless, direct evidence has not yet been reported which warrants definitive assignment of the sulfhydryl of a Cys residue as this nucleophile. In initial experiments with the pronase-derived FdUMP-CH_2-H_4folate-peptide (P_1), we did not detect half-cystines by amino acid analysis (6) and concluded that the covalent linkage to FdUMP could not involve Cys. In a recent abstract (21), Danenberg and Heidelberger reported that a pronase-derived peptide from [^{3}H]FdUMP-CH_2-H_4folate-[^{35}S]thymidylate synthetase contained equivalent amounts of ^{3}H and ^{35}S. Since this result implied the presence of Cys, amino acid compositions of peptides prepared in our laboratory were reinvestigated (Table I). For reasons unclear to us, analysis of hydrolyzed samples failed to definitively reveal Cys as half-cystine, but prior performic oxidation clearly indicated the presence of one residue of cysteic acid. Thus, we are in agreement with the prior report (21) that Cys is a component of the FdUMP-CH_2-H_4folate-peptide .

The sequence of the FdUMP-CH_2-H_4folate-peptide (P_1) has been determined to be Ala-Leu-Pro-Pro-(His,Cys)-Thr. Since His and Cys are known to be present from amino acid analysis, they were assigned to positions corresponding to the two open Edman cycles where no Dns-amino acids could be identified, but their relative positions are unknown. Thus, there are three amino acids which may be considered candidates for the nucleophilic catalyst: His, Cys and Thr.

A direct approach towards identifying the nucleophile would be to obtain a single amino acid residue attached to FdUMP. Chemical hydrolysis is unsuitable for this purpose since the linkage to FdUMP is completely cleaved under conditions necessary for peptide bond hydrolysis (6). Danenberg and Heidelberger (21) subjected their [^{3}H]FdUMP-H_4folate-[^{35}S]peptide to performic acid and acid hydrolysis and observed a spot (R_f 0.05) on paper chromatography which contained both labels. The conclusion that this product corresponds to FdUMP covalently attached to Cys should *not* be drawn since, as shown here, performic acid oxidation and acid hydrolysis results in conversion of Cys to cysteic acid; clearly if FdUMP is covalently bound to Cys in the peptide, such treatment would result in cleavage of this bond. Unfortunately, the bulky ligands attached near the C-terminus and the Leu-Pro-Pro sequence at the N-terminus block further digestion by the least specific exopeptidases available to us, and amino acids are not present which are susceptible to the common endopeptidases.

An alternative approach involves unequivocal elimination of two of the three candidate nucleophiles. The Pauly reagent forms chromophoric derivatives

with imidazoles (15-17) but not with N-substituted imidazoles (22). Since the peptide, P_1, quantitatively reacts with this reagent and a peptide possessing both monoazo-imidazole and FdUMP can be isolated, it is unlikely that the covalent linkage to FdUMP involves the His residue. Our previous report (23) that the FdUMP-CH_2-H_4folate-enzyme and the uncomplexed enzyme contain the same number of titrateable sulfhydryl groups is difficult to reconcile with the notion that Cys is the residue involved. However, should this result be incorrect or artifactual, Cys would be a prime candidate.

The reactive electrophilic species of CH_2-H_4folate is believed to be the 5- or 10-iminium ion (24,25); one of these undoubtedly exists on the reaction pathway of the normal enzymic reaction, and is a direct precursor to the FdUMP-CH_2-H_4folate-enzyme complex. Thus, one approach to distinguish the two possibilities is to establish whether H_4folate is attached to FdUMP *via* its 5- or 10-nitrogen. It is well known that H_4folates with a free 5-NH are extremely susceptible to oxidation, whereas 5-substituted derivatives are relatively stable (26). The fact that one of the peptides (P_4) used in this study was stable for over six months without precautions to avoid oxidation clearly indicates that the methylene group links FdUMP to the 5-nitrogen of H_4folate, and not the 10-nitrogen as suggested by other workers (4). Thus, in the formation of the FdUMP-CH_2-H_4folate-enzyme complex, it is reasonable to conclude that the 5-iminium ion of CH_2-H_4folate is the reactive electrophilic species; since the reaction appears to be so analogous to the normal enzymic reaction, this intermediate is probably also involved in TMP formation.

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