Identification of the Folate Binding Sites on the Escherichia coli T-protein of the Glycine Cleavage System*

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T-protein is a component of the glycine cleavage system and catalyzes the tetrahydrofolate-dependent reaction. To determine the folate-binding site on the enzyme, 14C-labeled methylenetetrahydropteroyl-tetraglutamate (5,10-CH2-H4PteGlu6) was enzymatically synthesized from methylenetetrahydrofolate (5,10-CH2-H4folate) and [U-14C]glutamic acid and subjected to cross-linking with the recombinant Escherichia coli T-protein using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, a zero-length cross-linker between amino and carboxyl groups. The cross-linked product was digested with lysylendopeptidase, and the resulting peptides were separated by reversed-phase high performance liquid chromatography. Amino acid sequencing of the labeled peptides revealed that three lysine residues at positions 78, 81, and 352 were involved in the binding with polyglutamate moiety of 5,10-CH2-H4PteGlu6. The comparable experiment with 5,10-CH2-H4folate revealed that Lys-81 and Lys-352 were also involved in cross-linking with the monoglutamate form. Mutants with single or multiple replacement(s) of these lysine residues to glutamic acid were constructed by site-directed mutagenesis and subjected to kinetic analysis. The single mutation of Lys-352 caused similar increase (2-fold) in K_m values for both folate substrates, but that of Lys-81 affected greatly the K_m value for 5,10-CH2-H4PteGlu6 rather than for 5,10-CH2-H4folate. It is postulated that Lys-352 may serve as the primary binding site to α-carboxyl group of the first glutamate residue nearest the p-aminobenzoic acid ring of 5,10-CH2-H4folate and 5,10-CH2-H4PteGlu6, whereas Lys-81 may play a key role to hold the second glutamate residue through binding to α-carboxyl group of the second glutamate residue.

The glycine cleavage system is a multienzyme complex composed of four proteins termed P-, H-, T-, and L-protein and catalyzes the oxidative cleavage of glycine, a major catabolic pathway of glycine in various organisms. T-protein is a folate-dependent enzyme that catalyzes the formation of ammonia and 5,10-CH2-H4folate from the aminomethyl intermediate bound to the lipoate cofactor of H-protein (reviewed in Ref. 1). T-protein should have the interaction or binding site(s) with H-protein, the folate cofactor, and possibly P-protein and the active site that directly participates in the cleavage of the aminomethyl intermediate. While the kinetic properties of the forward (2) and the reverse (3) reactions catalyzed by T-protein have been extensively studied, little is known in terms of specific amino acid residues of T-protein involved in binding or catalysis.

The primary structures of T-proteins from eight different species have been determined so far (4–10). The sequence alignment by computer program CLUSTAL W (11) indicates that there are relatively high identities between T-proteins from plants and animals (46–88%), but there are less identities between animal enzymes and the yeast and Escherichia coli enzymes (~30%). Thus, the regions well conserved among the eight species are very limited (see Fig. 1). A partial sequence homology of T-proteins to α subunit of corynebacterial sarcosine oxidase and rat dimethylglycine dehydrogenase has been reported (12). The homologous region was considered to be the tetrahydrofolate binding domain, since all these enzymes catalyze the formation of 5,10-CH2-H4folate from different one-carbon donors (12). Physiological folate substrates generally have a string of glutamate residues attached to the benzoyl moiety of the cofactor, and the polyglutamylation enhances the cofactor binding to folate-dependent enzymes (13–16). The interaction between the polyglutamate moiety and protein has been extensively investigated in thymidylate synthase (TS) that uses 5,10-CH2-H4folate as a substrate. Crystal structure analysis revealed that TS binds the polyglutamate moiety with a positively charged hydrophilic surface, where only the first glutamate residue (Glu-1) nearest the p-aminobenzoic acid ring is rigidly fixed. The lysine residue at the position 50 of E. coli TS (the numbering of amino acid residues corresponds to that of Lactobacillus casei TS), which is nearly invariant among all TS enzymes, participates in the binding to α-carboxyl group of Glu-1 (13).

As part of an overall effort to identify the domains of T-protein responsible for cofactor recognition, complex association, and catalysis, we investigated specific lysine residues involved in the binding with the glutamyl tail of folate derivatives. In the present study, we utilized a carbodiimide-mediated covalent attachment of 14C-labeled 5,10-CH2-H4PteGlu4 to lysine residues located at or near the folate-binding site of E. coli T-protein. Three lysine residues were identified, and their role was confirmed by replacing these residues with glutamic acid by site-directed mutagenesis.

EXPERIMENTAL PROCEDURES

Materials—[U-14C]glutamic acid (9.29 GBq/nmol) and NaN4CO3 (2.0 GBq/nmol) were obtained from Amersham Pharmacia Biotech. Restriction endonucleases and other DNA modifying enzymes were purchased from New England Biolabs, Roche Molecular Biochemicals, Toyobo (Tokyo, Japan), or Takara Shuzo (Shiga, Japan). Oligonucleotides were from Life Technologies, Inc. Folic acid, lysylendopeptidase,
and mutated residues are boxed.

3 centrifugation (8,000 g, Saccharomyces cerevisiae acid into 5,10-CH₂-H₄folate essentially as described by Bognar and

pellet was resuspended in 3 ml of buffer B (50 mM potassium phosphate, pooled and concentrated by 70% ammonium sulfate precipitation. The

fractions containing the FPGS activity were

acid (20 MBq/mmol) were employed as substrates. A unit of FPGS

The sonicate was centrifuged at 105,000 g, 1/2-inch disrupter horn in the pulse mode (output 8, 20% duty cycle).

cated at 0 °C four times for 5 min using a Branson Sonifier 250 with a

volumes of buffer A and eluted with a linear gradient of 0–500 mM KCl

equilibrated with buffer A. The column was washed with 10 column

was applied to a phosphocellulose column (P11, 2.5 cm, 80 cm, Whatman) 3 were determined spectrophotometrically using

itate was resuspended in 20 ml of buffer A (50 mM potassium phos-

supernatant was made 70% ammonium sulfate saturation. The precip-

dryness with a Speed Vac concentrator (Savant) and resolved in water.

After the determination of concentration, the material was immediately

KCl, and soni-

for 60 min, and the

295 nm. The collected peak fractions were immediately stored at

nate contained 100 m M Tris, 50 m M glycine buffer, pH 9.3, 0.4 m M

5,10-CH₂-H₄PteGlu₄, respectively, as described (17) and used for synthesis of

90 cm, Amersham Pharmacia Biotech) equilibrated with buffer

3 were reduced (17) and used for synthesis of

and N-hydroxysuccinimide were purchased from Wako Pure Chemicals

plasmid pGT3–8.1, a pEMBL vector carrying the

mate Synthetase—

expression and Purification of Lactobacillus casei Folylpolyglutamate—

Expression and Purification of Recombinant E. coli T-protein—

Preparation of 14C-labeled 5,10-CH₂-H₄PteGlu₄— The reaction mixture contained 100 mM Tris, 50 mM glycine buffer, pH 9.3, 0.4 mM 5,10-CH₂-H₄folate, 4 mM [U-14C]glutamic acid (195 MBq/mmol), 5 mM ATP, 10 mM MgCl₂, 200 mM KCl, 5 mM DTT, 65.5 mM formaldehyde, 0.01% bovine serum albumin, and 924 units of L. casei FPGS in a total volume of 4 ml. The mixture was incubated at 37 °C for 3 h, and then protein ingredients were removed by filtration with a Centricon-10 concentrator (Grace Japan). The filtrate was divided into three equal portions, and a portion was applied to a reversed-phase C₁₈ column (TSKgel ODS-120T, 4.6 × 250 mm, Tosoh, Tokyo, Japan) attached to a 655 HPLC system (Hitachi, Tokyo, Japan). The column was developed with stepwise gradients of acetonitrile using solution A (50 mM ammonium acetate, pH 7.1) and solution B (10% acetonitrile in solution A) at a flow rate of 1 ml/min. The absorbance of the effluent was monitored at 295 nm. The collected peak fractions were immediately stored at

-80 °C in small aliquots until use. The samples were lyophilized to dryness with a Speed Vac concentrator (Savant) and resolved in water. After the determination of concentration, the immediately was used for cross-linking with ET. Concentrations of the folate derivative were determined spectrophotometrically using ε₂₉₅ = 32.6 × 10³ M⁻¹ cm⁻¹, and mass spectrometry, and matrix-assisted laser desorption ionization/time-of-flight mass spectrometry.

Expression and Purification of Recombinant B. coli T-protein—DNA manipulations were accomplished by standard techniques (20). The pEGCV(−5') plasmid containing the gcv operon (7) was digested with EcoRV and self-ligated. The product, pTZE/ETEH, contains gcvT and pTZE/ETEH/MV1190. An NdeI was introduced adjacent to the codon for N-terminal Ala of gcvT gene in pTZE/ETEH by oligonucleotide-directed mutagenesis with the sense oligonucleotide 5'-TCAAATGAGGACATATGAGCACAAGACG-3' (the Ndel site is underlined and modified bases are shown in boldface letters) according to the method of Kunkel et al. (23) using a Bio-Rad kit. Creation of a BamHI site placed 21 bases downstream of the stop codon and deletion of the...
**Folate Binding Sites on E. coli T-protein**

### Table I

| Construct | Oligonucleotide* |
|-----------|------------------|
| T<sup>+</sup> | 5'-GTAAATGCGAAGAAGCCTTGTTTG-3' |
| K352E | 5'-GTAAGTGGCAGAATGCCTTTTGG-3' |
| K352Q | 5'-GTAAGTGGCAGAATGCCTTTTGG-3' |
| K352R | 5'-GTAAGTGGCAGAATGCCTTTTGG-3' |
| K35E | 5'-GCCATGCGGAGAAGCTCACACCGA-3' |
| K81E | 5'-GCCATGCGGAGAAGCTCACACCGA-3' |
| K78E/K81E | 5'-GCCATGCGGAGAAGCTCACACCGA-3' |
| K78E | 5'-GCCATGCGGAGAAGCTCACACCGA-3' |

* Modified bases are shown in boldface letters.

These oligonucleotides were used for *in vitro* mutagenesis according to the method of Kunkel et al. (23).

These oligonucleotides were employed as mutagenic reverse primer for the first round of polymerase chain reaction in the megaprimer method (25).

* Nd<sup>e</sup> site present originally in the coding region without altering the amino acid were simultaneously carried out with sense oligonucleotides, 5'-TTGGACCTGTCAGTGGCGGCAACGTCCAG-3' (the BamHI site is underlined and modified bases are shown in boldface letter) and 5'-CATGGTTCACATGACACATGTGCTGTTTGGG-3' (the modified base is shown in boldface letter), respectively. The resultant plasmid (pTZE/TETHb) was digested with Nd<sup>e</sup>I and BamHI, and the Nd<sup>e</sup>-BamHI fragment was cloned into pET3a (24). The nucleotide sequence of the resultant expression vector pET/ET was confirmed by nucleotide sequencing employing a 373 DNA sequencing system (Perkin-Elmer).

* E. coli BL21(DE3)pLyS cells (24) transformed with pET/ET were grown in 200 ml of LB medium containing 20 mg/ml ampicillin and 30 μg/ml chloramphenicol. Expression was induced by the addition of 25 μl isopropyl-β-D-thiogalactopyranoside at the start of the incubation. Cell-free extracts were prepared as described previously (7) with buffer D (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 10 μM p-aminomethyl-naphthylmethanesulfonyl fluoride) and heated in a boiling water bath for 2 min. The supernatant of the heated sample was subjected to column chromatographies on DEAE-Sepharose CL-6B (2.5 × 10 cm), hydroxyapatite (2.8 × 8 cm), and Sephacryl S-100 columns (2.6 × 90 cm) essentially as described for the purification of wild-type ET with minor modifications. The elution from the SEAH-Sepharose column was performed with a linear gradient of NaCl (0.28–0.45 mM in 500 ml of buffer D). The buffer employed in the hydroxyapatite column chromatography contained no DTT, and the Centriprep-10 concentrator was replaced by a Centrifrap-3 concentrator. The mobility of EH in crude extracts and in the sample from each purification step corresponded to that of lipoylated holoh-protein on SDS-PAGE, indicating that the condition described above led to the full lipoylation of overexpressed EH. The final preparation was more than 95% pure as judged by SDS-PAGE. The activity of H-protein was assayed as described (7).

### Location of Polyglutamate Binding Sites on E. coli T-protein—Construction, Expression, and Purification of Mutant E. coli T-protein

**Construction**—Construction of some mutant ETs (K352E, K352Q, K352R, K75E, K81E, and K75E/K81E) was performed by the megaprimer polymerase chain reaction mutagenesis procedure described by Sarkar and Sommer (25). For this purpose, pTZE/TETHb was trimmed by digestion with BamHI and self-ligated. The resulted plasmid was employed in the mutagenesis reaction with oligonucleotides listed in Table I. The Nd<sup>e</sup>-BamHI fragments were isolated from the mutant plasmids and ligated into pET3a. To construct K75E/K352E, K81E/K352E, or K75E/K81E/K352E mutant, each Nd<sup>e</sup>-EcoRI fragment (612 base pairs) from K352E mutant plasmid was ligated in a one-step reaction into pET3a. Construction of K75E, K78E/K81E, or K75E/K81E/K352E mutant was performed by the megaprimer polymerase chain reaction mutagenesis procedure described by Sarkar and Sommer (25). For the first round of polymerase chain reaction, pET/ET was used as a template with mutagenic primer, 5'-CATGCGGAGAAGCTCACACCGA-3'.
AGGG-3'. The double-strand polymerase chain reaction products were gel-purified and used as megaprimer in the second round of polymerase chain reaction along with T7 terminator primer, 5'-GCTAGTTATGCCTACACGCGTT-3', using either pET/ET (for K78E and K78E/K352E) or the expression plasmid for K352E (for K78E/K352E and K78E/K352E/K352E) as a template. Amplified products were purified, digested with NdeI and BamHI, and ligated into pET3a. After verification of the mutations by DNA sequencing, the expression plasmids were transfected into E. coli BL21(DE3)pLysS cells. Expression and purification of mutant ETs were carried out as described for the recombinant wild-type ET.

**Kinetic Analysis**—Apparent $K_m$ values of mutant ETs for 5,10-CH$_2$H$_4$folate and 5,10-CH$_2$H$_4$PteGlu$_4$ were determined at varied concentrations of either folate substrates with a constant concentration of other two substrates using the "coupled assay" (3) with some modifications. The reaction mixture contained in 0.25 ml: 50 mM Tris-HCl, pH 8.5, 10 mM DTT, 0.25 mM pyridoxal phosphate, 0.8 mM NaH$_4$CO$_3$ (37 MBq/mmol), 1.2 mM formaldehyde, 50 units of E. coli P-protein, 32 μg of diaphorase (pig heart, Roche Molecular Biochemicals), and T-protein. The reaction was initiated by the addition of reduced EH, continued for 3 min at 37 °C, and terminated by the addition of 12.5 μl of 50% perchorlic acid. The mixture was bubbled with CO$_2$ and the remaining radioactivity was determined by scintillation counting. The concentration of 5,10-CH$_2$H$_4$folate was 40–360 nM for the experiments with wild-type ET, K352E, K352Q, K352R, K75E, K75R, K81E, and K78E/K352E. The data are expressed as the percentage of incorporated radioactive glutamate residues. 5,10-CH$_2$H$_4$PteGlu$_4$ prepared from chemically synthesized pteroyltetrahydrofolate was used as a standard.

**Other Methods**—Protein concentration was routinely determined by the method of Bradford (26) with bovine serum albumin as a standard. Concentrations of purified wild-type and mutant ETs and EH were also estimated by amino acid analysis using a Hitachi 830 amino acid analyzer. Comparison of the data obtained by Bradford method and the amino acid analysis revealed that the amounts of proteins estimated with Bradford method were somewhat greater than those obtained by amino acid analysis. The peaks P1, P2, and P4 were radioactive and not observed in the control experiment. The another radioactive peak was assigned to the incorporated radioactive glutamate residues. 5,10-CH$_2$H$_4$PteGlu$_4$ prepared from chemically synthesized pteroyltetrahydrofolate gave the same retention time as P2 (data not shown). P1 and P2 gave the same cross-linked lysylpeptides when used to cross-linking with ET. In addition, both P1 and P2 served as a substrate for T-protein. In the following cross-linking experiments, P2 fraction was employed as 14C-labeled 5,10-CH$_2$H$_4$PteGlu$_4$.

**Localization of the Folate Binding Sites by Cross-linking and Lysylpeptide Mapping**—Wild-type ET was preincubated with 14C-labeled 5,10-CH$_2$H$_4$PteGlu$_4$, and the resultant peptides were separated by HPLC (Fig. 3B). As a control, ET alone was treated with the cross-linkers and subjected to lysylpeptide mapping (Fig. 3A) and Edman sequencing. The peaks P1, P2, and P4 were radioactive and not observed in the control experiments. The other radioactive peak, P3, was inseparable from peak P', and the absorbance at 220 nm was greater than that of P' (Fig. 3, A and B). The initial unnamed radioactive peak (Fig. 3B) did not correspond to any radioactive peaks. The incorporated radioactive glutamate residues were cleaved at the C-terminal side of the lysyl residues not detected in the above Edman degradation. Namely, peptide P1 was cleaved to peptides 76–78 (K2, Fig. 1) and 79–81 (K3, Fig. 1), peptide P2 to peptides 350–352 (K12, Fig. 1) and 353–360 (K13, Fig. 1), and peptide P4 to peptides 79–81 and 82–119 (K4, Fig. 1). The cleavage of P2 was incomplete due to the resistance of the lysyl-proline bond to 352–353 to lysylendopeptidase (Fig. 3A). K2 and K3 were eluted from the C$_{18}$ column in the void volume fraction and K4 was eluted.

**Mass Spectrometry**—The mass spectroscopic analysis of folylpolyglutamates separated by a reversed-phase C$_{18}$ column was performed by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry using a Voyager RP mass spectrometer (PerSeptive Biosystems). The samples containing ~3.6 pmol of folate were employed and 1% α-cyano-4-hydroxyquinamic acid in 33% acetonitrile containing 0.1% trifluoroacetic acid was employed as a matrix. Des-Arg$^1$bradykinin (9.04 ± 1 Da) and potassium iodide (39.00 Da) were used as external standards.

**RESULTS**

**Preparation of 14C-labeled 5,10-CH$_2$H$_4$PteGlu$_4$—14C-labeled 5,10-CH$_2$H$_4$folate and 1-14C]glutamic acid using purified recombinant L. casei FPGS, and the products were separated by HPLC on a reversed-phase C$_{18}$ column. Formaldehyde was added to the reaction mixture to prevent the release of methylene-carbon from the folate substrate during incubation. The product and substrate were separated by stepwise gradient elution in acetonitrile in 50 mM ammonium acetate, pH 7.1 (Fig. 2). Four peak fraction showing characteristic absorption spectra of 5,10-CH$_2$H$_4$folate with maximum at around 295 nm were obtained, and their properties were summarized in Table II. Molecular masses of P1 and P2 were in good agreement with that of 5,10-CH$_2$H$_4$PteGlu$_4$ (844,298 ± 1 Da), whereas those of P3 and P4 were consistent with that of 5,10-CH$_2$H$_4$folate (457,171 ± 1 Da). These results agree well with the amounts of...
Evidence for the modification of amino acid residues was separated by HPLC. No radioactive peak was detected and no glutamic acid C-terminal cleavage, and the peptides were cross-linked products. A type ET cross-linked with 5,10-CH₂-H₄PteGlu₁⁴C; ET cross-linked with 5,10-CH₂-H₄folate.

Cross-linking experiment with 5,10-CH₂-H₄folate gave cross-linked peptide peaks P5 and P6 (Fig. 3C), which had the same amino acid sequence as P2 and P4, respectively. The results indicate that Lys-352 and Lys-81 were also involved in the coupling with the carboxyl group(s) of the monoglutamate residue. Peptide peaks corresponding to P1 and P3 were not detected (Fig. 3C).

ET with covalently cross-linked 5,10-CH₂-H₄folate has no significant enzymatic activity with or without externally added folate substrate.

Effects of the Mutations of the Folate Binding Sites—To assess the contribution for interaction with folate substrate, above three lysine residues (Lys-78, Lys-81, and Lys-352) and Lys-75 were subjected to site-directed mutagenesis. Lys-352, the residue absolutely conserved among T-proteins from eight various species (Fig. 1), was mutated to glutamic acid, glutamic acid, or arginine. Other lysine residues were replaced with glutamic acid. In addition to the replacement of a single residue, multiple replacements with glutamic acid were carried out. All mutant proteins were highly expressed in E. coli BL21(DE3)pLysS cells as soluble protein and purified by the same strategy used for the wild-type enzyme. The purified proteins gave a single band on SDS-PAGE (not shown). Apparent $K_m$ values of mutant ETs for 5,10-CH₂-H₄folate and 5,10-CH₂-H₄PteGlu₄ were determined and summarized in Table IV. Wild-type ET showed 6.5-fold higher affinity for 5,10-CH₂-H₄PteGlu₄ than that for 5,10-CH₂-H₄folate. The single mutation of Lys-352 to Glu and Gln increased $K_m$ values for both folate substrates about 2-fold, whereas mutation to Arg caused no effect. Thus, the side chain positive charge appears to be essential for the interaction. Mutation of Lys-78 to Glu increased $K_m$ values for both folate substrates 1.4-fold. The K81E mutant showed a 3-fold increase in $K_m$ for 5,10-CH₂-H₄folate and a 16-fold increase in $K_m$ for 5,10-CH₂-H₄PteGlu₄. The contribution of Lys-75 was not evidenced by the cross-linking experiment but the mutation of Lys-75 to Glu increased $K_m$ in 5,10-CH₂-H₄PteGlu₄ 8-fold. The multiple mutations resulted in a 7–13-fold increase in $K_m$ values for 5,10-CH₂-H₄folate and a 5–252-fold increase in $K_m$ values for 5,10-CH₂-H₄PteGlu₄. Mutation of above lysine residues affected slightly the affinity of ET for the protein substrate EH. $K_m$ values for EH of wild-type ET and the mutant K75E/K78E/K81E/K353E were 0.45 $\mu$M and 0.53 $\mu$M, respectively. Changes in $K_m$ values were not so significant, and even higher values were obtained (Table IV).

**DISCUSSION**

In this study, ¹⁴C-labeled 5,10-CH₂-H₄PteGlu₄ was used to investigate the folate binding sites on E. coli T-protein. 5,10-CH₂-H₄PteGlu₄ whose terminal three glutamate residues were uniformly labeled with ¹⁴C was enzymatically synthesized from 5,10-CH₂-H₄folate and [U-¹⁴C]glutamic acid using L. casei FPGS. The L. casei enzyme was chosen because of its preference for 5,10-CH₂-H₄folate as the substrate among various folates (21). The radiolabeled products separated by HPLC were solely tetraglutamate derivatives, and no other polyglutamate forms were obtained under the conditions used here (Table II). The products were separated into two peak fractions by HPLC (P1 and P2, Fig. 2), but they behaved similarly as a substrate for ET and gave the same cross-linked lysylpeptides next to P4 (Fig. 3, A and B). These results indicate that the above three lysine residues could not be detected in the Edman degradation due to the modification and, therefore, are likely the candidates for the cross-linking residues with the polyglutamate moiety of 5,10-CH₂-H₄PteGlu₄.

Peaks P1 (Fig. 3A) contained peptides K1, K5, and K7. N-terminal sequencing of P3 up to 20 residues showed the existence of peptide K6–7 whose Lys-157 was not modified, in addition to the three peptides mentioned. P3 was digested with S. aureus V8 protease under the specific condition for glutamic acid C-terminal cleavage, and the peptides were separated by HPLC. No radioactive peak was detected and no evidence for the modification of amino acid residues was obtained by amino acid sequencing of all the V8 peptides (data not shown). Peptide K6–7 with intact Lys-157 was not yielded from the control experiment and from the ET cross-linked with 5,10-CH₂-H₄folate. Therefore, the inability of lysylendopeptidase to cleave the C-terminal side of Lys-157 may be attributed to some kind of interaction between Lys-157 and 5,10-CH₂-H₄PteGlu₄.

The parallel cross-linking experiment with 5,10-CH₂-H₄folate gave cross-linked peptide peaks P5 and P6 (Fig. 3C), which had the same amino acid sequence as P2 and P4, respectively. The results indicate that Lys-352 and Lys-81 were also involved in the coupling with the carboxyl group(s) of the monoglutamate residue. Peptide peaks corresponding to P1 and P3 were not detected (Fig. 3C).

Fig. 3. 

**HPLC separation of lysylendopeptidase digests of cross-linked products.** A, wild-type ET treated with EDC; B, wild-type ET cross-linked with 5,10-CH₂-H₄PteGlu[¹⁴C]Glu₄; C, wild-type ET cross-linked with 5,10-CH₂-H₄folate ---, absorbance at 220 nm; ---, CH₃CN (%), ---, radioactivity.
when cross-linked with ET. The differences of these two fractions were not investigated further.

EDC was employed to produce zero-length cross-linking between contacted carboxyl groups of polylglutamate moiety of 5,10-CH₂-H₄PteGlu₄ and amino groups of ET in the binary complex. Binding of EDC-activated folate derivatives to folate-depended enzymes has been previously reported with thymidylate synthase (14). In a preliminary experiment, we activated 5,10-methenyltetrahydrofolate synthetase (13–16). Mutation of Lys-352 or Glu-1 residue among all TS enzymes. Lys-352 of ET is conserved among T-proteins so far studied, whereas Lys-78 and Lys-81 are not (Fig. 1). Considering these circumstances, it is tempting to postulate that Lys-352 interacts with the charged hydrophilic surface of the protein where Glu-1 is rigidly fixed through its carboxyl group with Lys-50, a nearly invariant residue among all TS enzymes. Lys-352 of ET is conserved among T-proteins so far studied, whereas Lys-78 and Lys-81 are not (Fig. 1). Considering these circumstances, it is tempting to postulate that Lys-352 interacts with the charged hydrophilic surface of the protein where Glu-1 is rigidly fixed through its carboxyl group with Lys-50, a nearly invariant residue among all TS enzymes. Lys-352 of ET is conserved among T-proteins so far studied, whereas Lys-78 and Lys-81 are not (Fig. 1). Considering these circumstances, it is tempting to postulate that Lys-352 interacts with the charged hydrophilic surface of the protein where Glu-1 is rigidly fixed through its carboxyl group with Lys-50, a nearly invariant residue among all TS enzymes.

The contribution of these lysine residues to the binding of the folate substrates with ET was confirmed by site-directed mutagenesis and kinetic analysis. Kinetic analyses were conducted employing saturated amounts of H-, P-, and L-protein (diaphorase), 5,10-CH₂-H₄PteGlu₄ was better substrate for wild-type ET than 5,10-CH₂-H₄folate (Table IV), in good agreement with other folate-dependent enzymes (13–16). Mutation of Lys-352 or

\[
\begin{array}{cccccccc}
\text{Cycle} & \text{Amino acid} & \text{Position} & \text{Yield} & \text{Amino acid} & \text{Position} & \text{Yield} & \text{Amino acid} & \text{Position} & \text{Yield} \\
1 & L & 76 & 18.9 & V & 350 & 136 & S & 79 & 27.3 \\
2 & T & 77 & 9.1 & T & 351 & 53.3 & G & 80 & 75.0 \\
3 & ND & ND & ND & A & 82 & 92.9 & Y & 84 & 84.6 \\
4 & S & 79 & 4.1 & P & 353 & 55.0 & L & 83 & 75.3 \\
5 & G & 80 & 13.1 & V & 354 & 61.8 & N & 87 & 61.5 \\
6 & K & 81 & 9.6 & F & 355 & 62.5 & M & 85 & 16.0 \\
7 & & & & V & 356 & 75.0 & & & \\
8 & & & & & & & & & \\
9 & & & & & & & & & \\
10 & & & & & & & & & \\
11 & K & 360 & 5.4 & & & & & & \\
\end{array}
\]

\( ^a \text{ND, not detected.} \)

\[
\text{Table III}
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Edman sequencing of the radioactive lysylpeptides in Fig. 3B

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\begin{array}{cccccccc}
\text{Cycle} & \text{Amino acid} & \text{Position} & \text{Yield} & \text{Amino acid} & \text{Position} & \text{Yield} & \text{Amino acid} & \text{Position} & \text{Yield} \\
1 & L & 76 & 18.9 & V & 350 & 136 & S & 79 & 27.3 \\
2 & T & 77 & 9.1 & T & 351 & 53.3 & G & 80 & 75.0 \\
3 & ND & ND & ND & A & 82 & 92.9 & Y & 84 & 84.6 \\
4 & S & 79 & 4.1 & P & 353 & 55.0 & L & 83 & 75.3 \\
5 & G & 80 & 13.1 & V & 354 & 61.8 & N & 87 & 61.5 \\
6 & K & 81 & 9.6 & F & 355 & 62.5 & M & 85 & 16.0 \\
7 & & & & V & 356 & 75.0 & & & \\
8 & & & & & & & & & \\
9 & & & & & & & & & \\
10 & & & & & & & & & \\
11 & K & 360 & 5.4 & & & & & & \\
\end{array}
\]

\( ^a \text{ND, not detected.} \)

\[
\text{Table IV}
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Kinetic constants of wild-type and mutant E. coli T-protein

\[
\begin{array}{cccccccc}
\text{T-protein} & \text{Km}_{\text{5,10-CH₂-H₄folate}} & \text{Km}_{\text{5,10-CH₂-H₄PteGlu₄}} & \text{kcat}_{\text{5,10-CH₂-H₄folate}} & \text{kcat}_{\text{5,10-CH₂-H₄PteGlu₄}} & \text{kcat/Km}_{\text{5,10-CH₂-H₄folate}} & \text{kcat/Km}_{\text{5,10-CH₂-H₄PteGlu₄}} \\
\text{Wild type} & 67.7 & 14.4 & 0.213 & 10.4 & 8.75 & 0.841 \\
\text{K352E} & 136 & 9.98 & 0.073 & 21.1 & 9.46 & 0.448 \\
\text{K352Q} & 104 & 9.49 & 0.091 & 18.6 & 10.9 & 0.556 \\
\text{K352R} & 533 & 4.25 & 0.019 & 9.74 & 6.79 & 0.679 \\
\text{K75E} & 166 & 12.7 & 0.077 & 82.9 & 17.9 & 0.216 \\
\text{K75E/K352E} & 660 & 16.1 & 0.024 & 396 & 18.7 & 0.491 \\
\text{K78E} & 95.6 & 12.6 & 0.132 & 14.9 & 8.36 & 0.351 \\
\text{K81E} & 216 & 13.6 & 0.063 & 164 & 18.7 & 0.114 \\
\text{K78E/K352E} & 480 & 11.9 & 0.025 & 396 & 23.5 & 0.059 \\
\text{K81E/K352E} & 172 & 11.1 & 0.065 & 49.8 & 12.2 & 0.245 \\
\text{K78E/K81E/K352E} & 533 & 17.3 & 0.032 & 1030 & 33.8 & 0.033 \\
\text{K78E/K81E} & 263 & 17.7 & 0.067 & 164 & 25.0 & 0.089 \\
\text{K78E/K81E/K352E} & 660 & 16.1 & 0.024 & 1540 & 30.1 & 0.020 \\
\text{K78E/K81E} & 474 & 14.8 & 0.031 & 804 & 25.1 & 0.031 \\
\text{K75E/K78E/K81E/K352E} & 893 & 15.7 & 0.018 & 2620 & 17.2 & 0.007 \\
\end{array}
\]
substrates may reflect the impaired binding ability to Glu-2 of 5,10-CH₂-H₄PteGlu₄.

As mentioned above, negatively charged glutamate residues of folyopolyglutamate lie in a position where they can interact with surface region of E. coli TS having positive potential (13). Maras et al. (14) suggested that a region containing multiple Lys and Arg residues serves as a polyglutamate binding site of rabbit liver 5,10-methenyltetrahydrofolate synthetase. Therefore, we investigated the role of Lys-75 situated in the vicinity of Lys-78 and Lys-78 are implicated in interaction with polyglutamate tail of mutants containing K78E mutation suggests that Lys-75 and the present cross-linking study provides the first evidence of action of T-protein with the folate substrates require further glutamate residues of polyglutamate tail (13). Details of interconnection, it is worth noting that there is considerable variance in folate-binding sites in T-proteins from other species. In this study seems to be specific for ET, and there must be distinct multifunctional lysyl residues of ET, however, are not situated in the region of T-protein they indicated.

Lys-75, -78, and -81 are not conserved in other T-proteins; therefore, the putative folate-binding site discussed in this study seems to be specific for ET, and there must be distinct folate-binding sites in T-proteins from other species. In this connection, it is worth noting that there is considerable variability in the region of TS that contacts the second and third glutamate residues of polyglutamate tail (13). Details of interaction of T-protein with the folate substrates require further elucidation; crystal structure analysis is essential. However, the present cross-linking study provides the first evidence of the folate binding sites on T-protein.

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