Probing the H-protein-induced Conformational Change and the Function of the N-terminal Region of *Escherichia coli* T-protein of the Glycine Cleavage System by Limited Proteolysis*

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T-protein, a component of the glycine cleavage system, catalyzes a tetrahydrofolate-dependent reaction. Previously, we reported a conformational change of *Escherichia coli* T-protein upon interacting with *E. coli* H-protein (EH), showing an important role for the N-terminal region of the T-protein in the interaction. To further investigate the T-protein catalysis, the wild type (ET) and mutants were subjected to limited proteolysis. ET was favorably cleaved at Lys81, Lys154, Lys288, and Lys352 by lysylendopeptidase and the cleavages at Lys81 and Lys154 were strongly prevented by EH. Although ET was highly resistant to trypsinolysis, the mutant with an N-terminal 7-residue deletion (ETΔ7) was quite susceptible and instantly cleaved at Arg16 accompanied by the rapid degradation of the resulting C-terminal fragment, indicating that the cleavage at Arg16 is the trigger for the C-terminal fragmentation. EH showed no protection from the N-terminal cleavage, although substantial protection from the C-terminal fragmentation was observed. The replacement of Leu6 of ET with alanine resulted in a similar sensitivity to trypsin as ETΔ7. These results suggest that the N-terminal region of ET functions as a molecular “hasp” to hold ET in the compact form required for the proper association with EH. Leu6 seems to play a central role in the hasp function. Interestingly, Lys352 of ET was susceptible to proteolysis even after the stabilization of the entire molecule of ET by EH, indicating its location at the surface of the ET-EH complex. Together with the buried position of Lys81 in the complex and previous results on folate binding sites, these results suggest the formation of a folate-binding cavity via the interaction of ET with EH. The polyglutamyl tail of the folate substrate may be inserted into the bosom of the cavity leaving the pteridine ring near the entrance of the cavity in the context of the catalytic reaction.

The glycine cleavage system is a multienzyme complex composed of four proteins, P-, H-, T-, and L-protein, which catalyzes the reversible oxidation of glycine yielding carbon dioxide, ammonia, 5,10-CH2-H4folate, and a reduced pyridine nucleotide. T-protein is a folate-dependent enzyme that catalyzes the release of ammonia and the transfer of the methylene carbon unit to H4folate from the intermediate attached to H-protein after the decarboxylation of glycine catalyzed by P-protein (reviewed in Ref. 1). The kinetic properties of the forward (2) and the reverse (3) reaction catalyzed by T-protein have been studied extensively, and the primary structures of T-proteins from eight species have been determined (4–10) as well as many putative T-protein sequences derived from genome analyses.

We found previously that lysine residues of *Escherichia coli* T-protein (ET) at positions 78, 81, and 352 are involved in the interaction with the polyglutamate moiety of 5,10-CH2-H4PteGlu4 (11). From the results of a kinetic analysis with single and multiple substitution mutants for these lysine residues, it was postulated that Lys81 interacts with the α-carboxyl group of the first glutamate residue nearest the p-amino-nobenoic acid ring of 5,10-CH2-H4PteGlu4 and that Lys81 interacts with the α-carboxyl group of the second glutamate residue. Lys78 seems to participate in the binding of the peripheral glutamate residues. Lys352 is conserved among the T-proteins studied thus far, whereas Lys81 and Lys78 are not. Therefore, other lysine residues must be responsible for the binding of the second and third glutamate residue of 5,10-CH2-H4PteGlu4 in T-proteins from other species. Furthermore, a cross-linking study (12) revealed that the interaction of *E. coli* H-protein (EH) with ET causes a conformational change of ET and results in intramolecular cross-linking between Asp35 and Lys216 of ET. Intermolecular cross-linking between Lys288 of ET and Asp13 of EH was also identified, indicating the participation of the region in the interaction between ET and EH. The extreme N-terminal region of ET is essential for the interaction with EH because such intramolecular and intermolecular cross-linking was not observed in a complex composed of the N-terminal 7-residue deletion mutant of ET (ETΔ7) and EH. The marked decrease in the kcat/Km value of ETΔ7 for EH compared with the wild type enzyme exhibited a good correlation with the cross-linking results.

In the present study, we used limited proteolysis to further examine the function of the N-terminal region as well as the mobile nature of the T-protein catalysis. Two amino acid residues, Thr1 and Leu6, that are conserved in all eight T-proteins were mutated separately to alanine. The resulting mutants (ETT4A and ETTL6A), ET, and ETΔ7 were overexpressed as His6-tagged proteins at the C-terminal end, purified, and subjected to proteolysis. The profile of protease digestion of these proteins in the absence or presence of substrates suggested that the functional lysine residues of ET identified previously as preferred sites for cleavage by trypsin and lysylendopeptidase. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: 5,10-CH2-H4folate, methylenetetrahydrofolate; 5,10-CH2-H4PteGlu4, methylenetetrahydropteroyltetrahydrofolate; EH, *E. coli* H-protein; Lys-C, lysylen-dopeptidase; TPCK-trypsin, L-1-tosylamino-2-phenylethyl chloromethyl ketone-treated trypsin.
are protease-accessible and that some of them are buried in the ET-EH complex. Moreover, it is revealed that the N-terminal region serves as a molecular "harp" to hold the ET molecule in the proper folding critical for association with EH. Leu6 plays a pivotal role in the harp function. The formation of the folate binding cavity by the interaction of ET with EH was also probed by limited proteolysis.

EXPERIMENTAL PROCEDURES

Materials—([14C]Sodium bicarbonate was obtained from Amersham Biosciences. Restriction endonucleases and other DNA modifying enzymes were purchased from New England Biolabs, Roche Molecular Biologies (Indianapolis, IN), New England BioLabs (Tokyo, Japan), or Takara Shuzo (Kyoto, Japan). Oligonucleotides were from Hokkaido System Science (Sapporo, Japan). t-1-Tosylamide-2-phenylethyl chloromethyl ketone-treated bovine pancreatic trypsin (TPCK-trypsin) was purchased from Worthington, and soybean trypsin inhibitor was from Sigma. Lysylendopeptidase (Lys-C) and folic acid were obtained from Wako Pure Chemicals (Osaka, Japan). Pteroyltetraglutamate from Schircks Laboratory (Jona, Switzerland) and folic acid were reduced by the method of Kisliuk (13) and used for the synthesis of 5,10-CH2-H4PteGlu4 and 5,10-CH2-H4folate, respectively, as described (14). Recombinant E. coli P-protein and H-protein were prepared as described previously (11).

Expression and Purification of Wild Type and Mutant E. coli T-protein—Tag at the C-terminal End—DNA manipulations were accomplished using standard techniques (15). To construct plasmids for the expression of ET and ETΔ7 with a His6 tag at the C-terminal end, pET3a with an insert of ET DNA (pET/ET) (11) or ETΔ7 DNA (12) was cut with EcoRI and employed in a polymerase chain reaction with oligonucleotides 5'-TAATACGACCATATAGGGT-3' (TT promoter primer) as 5'-primer and 5'-AAAGCTTTCGACCCGCGACGTTTCCG3'-3 (the XhoI site is underlined) as 3'-primer. The products were purified with a QIAEX II kit (Qiagen) after agarose gel electrophoresis, digested with NdeI and XhoI, and ligated into pET23b. The nucleotide sequences of the resultant plasmids, pET23b/ET and pET23b/ETΔ7, were confirmed with a 373 DNA sequencing system (Applied Biosystems). For the introduction of two mutations, ETΔ7 and ETΔ7A, polymerase chain reaction was carried out using EcoRI-digested pET/ET as a template with the respective 5'-primer and 5'-primer plus NdeI and ligated into pET23b.

The nucleotide sequences of the resulting plasmids, pET23b/ET and pET23b/ETΔ7, were confirmed by DNA sequencing. E. coli BL21(DE3)pLysS cells (16) transformed with the constructed pET23b/ET or pET23b/ETΔ7 were grown in 100 ml of LB medium containing 20 μg/ml ampicillin and 25 μg/ml chloramphenicol at 30 °C for 2 h. Expression was induced by 25 μM isopropyl-β-D-thiogalactosidase added at the start of the incubation. Cell-free extracts were prepared as described previously (7) with buffer A (20 mM potassium phosphate, pH 7.4, 0.3 mM NaCl, and 0.2 mM dithiothreitol) containing 20 mM imidazole and subjected to affinity chromatography on a Ni2+ column (1.5 × 2.2 cm, Qiagen). The samples were eluted with buffer A containing 50 mM imidazole, concentrated with a Centricon-10 (Millipore) and subjected to size exclusion chromatography on a Superdex 200 10/30 column (Amersham Biosciences) equilibrated with 50 mM Tris-HCl, pH 8.0, 0.2 mM NaCl, and 1 mM dithiothreitol. Two main peaks corresponding to molecular masses of 54 and 28 kDa were obtained. The former peak, containing a 39-kDa band verified by SDS-PAGE, was concentrated using Microcon-3 (Millipore), and the solvent was replaced with 50% acetonitrile containing 0.1% trifluoroacetic acid by repeated concentration. The sample solution was mixed with a saturated solution of sinapinic acid (3,5-dimethoxy-4-hydroxyphenylacetic acid) in 30% acetonitrile and 0.1% trifluoroacetic acid. The spectra of positive ions were recorded in the linear mode on a Voyager-DE STR mass spectrometer (Perseptive Biosystems) equipped with a delayed extraction device. External calibration was performed with Sequazyme Peptide Mass Standard Kit Mix 3 (Perseptive Biosystems) containing insulin (bovine), thioredoxin (E. coli), and cytochrome c (horse) with a mass (M + H)+ of 2749.59, 11,674.48, and 16,952.56 Da, respectively.

Other Methods—Protein concentrations were routinely determined by the method of Bradford (18) with bovine serum albumin as a standard. Purification of concentrated wild type and mutant ETs with a His6 tag at the C terminus and EH were estimated as described previously (11).

RESULTS

Expression and Purification of Wild Type and Mutant E. coli T-protein with a His6 Tag at the C-terminal End—To facilitate purification and to obtain mutant ETs without contamination by wild type ET originating from the host cell gene, we introduced a His6 tag at the C-terminal end of the ETs with two extra amino acids (–LEH). Four proteins, termed ET-His6, ETΔ7-His6, ETΔ7A-His6, and ETΔ6A-His6, were highly expressed in E. coli as soluble forms and purified by Ni2+ affinity column chromatography and subsequent gel filtration. The final preparation of each protein gave a single band on SDS-PAGE (not shown). Purified ET-His6 showed comparable specific activity (16,885 units/mg) to ET (15,127 units/mg) (12), indicating no significant effect of the C-terminal extension on the activity. The specific activity of purified ETΔ7-His6 (906 units/mg) was about 5% relative to ET-His6, again giving a result comparable to ETΔ7 (764 units/mg) (12). T4A and L6A mutations led to a reduction in the specific activity to 43% (722 units/mg) and 79% (13,364 units/mg), respectively, relative to wtET-His6.

Kinetic Analysis—Steady state kinetic studies were carried out by varying the concentration of one substrate and keeping the concentrations of the other two substrates constant (Table

Expression of E. coli T-protein by Limited Proteolysis

Analysis of E. coli T-protein by Limited Proteolysis
I). ET-His<sub>6</sub> and ETΔ7-His<sub>6</sub> gave parameters comparable to those of ET and ETΔ7, respectively (12), showing a notable decrease (165-fold) in the k<sub>cat</sub>/K<sub>m</sub> value of ETΔ7-His<sub>6</sub> for reduced EH. ETT4A-His<sub>6</sub> and ETL6A-His<sub>6</sub> also exhibited a significant decrease (4- and 5-fold, respectively) in the k<sub>cat</sub>/K<sub>m</sub> value for reduced EH compared with ET-His<sub>6</sub>. These results suggest that both residues play an important role in the function of the N-terminal region of ET.

Protease Digestion.—To investigate the effect of the N-terminal mutation, the purified proteins were subjected to limited proteolysis by TPCK-trypsin (an ETs/trypsin ratio of 100:1), and the resulting peptides were analyzed by SDS-PAGE, Edman sequencing, assay of the activity, and/or mass spectrometry. ET-His<sub>6</sub> was resistant to trypsin digestion as revealed by the presence of about half of the intact ET band (Fig. 1A, lane 7) and the retention of about half of the original activity (Fig. 3A) even after a 4-h digestion. However, ETΔ7-His<sub>6</sub> was quite susceptible to trypsinolysis and converted to a 37-kDa fragment (F1) within the first 5 min of digestion (Fig. 1B, lane 2). F1 gave the N-terminal sequence starting from Met<sup>17</sup> (Table II) and retained the His<sub>6</sub> tag at the C-terminal end as probed by the antibody against the His<sub>6</sub> tag sequence in Western blotting (not shown). Loss of the N-terminal residues up to Arg 16 resulted in a complete loss of activity (Fig. 3B). F1 was rapidly degraded to small pieces through intermediary fragments of 20, 14, and 7 kDa (named F2, F3, and F4, respectively) (Fig. 1B, lanes 2–5). Consequently, the cleavage at Arg<sup>16</sup> seems to trigger the subsequent C-terminal fragmentation. The intermediate peptides were subjected to Edman sequencing, and the five N-terminal residues were determined as Met<sup>17</sup>–His<sup>21</sup>, Ala<sup>82</sup>–Gly<sup>86</sup>, and Met<sup>17</sup>–His<sup>21</sup> for F2, F3, and F4, respectively (Fig. 1B, lanes 2–5). treatments derived from wild type and mutant E. coli T-proteins

### Table I

<table>
<thead>
<tr>
<th>T-protein</th>
<th>Reduced EH</th>
<th>5,10-CH&lt;sub&gt;2&lt;/sub&gt;-H&lt;sub&gt;4&lt;/sub&gt;folate</th>
<th>5,10-CH&lt;sub&gt;2&lt;/sub&gt;-H&lt;sub&gt;4&lt;/sub&gt;PteGlu&lt;sub&gt;4&lt;/sub&gt;</th>
<th>NH&lt;sub&gt;4&lt;/sub&gt;Cl</th>
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</tr>
<tr>
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<td>19.4</td>
<td>28.1</td>
<td>18.4</td>
</tr>
<tr>
<td>ETΔ7-His&lt;sub&gt;6&lt;/sub&gt;</td>
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<td>2.9</td>
<td>0.17</td>
<td>151</td>
</tr>
<tr>
<td>ETT4A-His&lt;sub&gt;6&lt;/sub&gt;</td>
<td>1.20</td>
<td>8.5</td>
<td>7.08</td>
<td>86.9</td>
</tr>
<tr>
<td>ETL6A-His&lt;sub&gt;6&lt;/sub&gt;</td>
<td>2.37</td>
<td>13.3</td>
<td>5.61</td>
<td>87.5</td>
</tr>
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</table>

Fig. 1. Time course of trypsin digestion of E. coli T-proteins. E. coli T-proteins were digested with trypsin at an ET:trypsin (w/w) ratio of 100:1 as described under “Experimental Procedures.” Digests containing 3.3 μg of ET were loaded for SDS-PAGE (16.5% T and 3% C acrylamide gels) (17) and stained with Coomassie Blue. A, ET-His<sub>6</sub>; B, ETΔ7-His<sub>6</sub>; C, ETT4A-His<sub>6</sub>; D, ETL6A-His<sub>6</sub>. The digestion times in minutes are indicated below the lanes, and the standard protein markers (lanes M1 and M2) are on the left. The fragments derived from ETΔ7-His<sub>6</sub> are termed as indicated on the right.
is conceivable that random cleavage occurs at several positions in the C-terminal one-third of ETΔ7-His$_6$ after the removal of 16 residues from the N-terminal. ETL6A-His$_6$ behaved similar to ETΔ7-His$_6$ (Fig. 1D) in trypsinolysis, indicating a central role for the leucine residue in the function of the N-terminal region. Arg$^{16}$ of ETT4A-His$_6$ was somewhat resistant to trypsinolysis (Fig. 1C).

The effects of substrate binding on susceptibility to trypsinolysis were examined in the presence of EH (a 2-fold molar excess over ETs) or 5,10-CH$_2$-H$_4$PteGlu$_4$. In these experiments, TPCK-trypsin was employed at an ETs/trypsin (w/w) ratio of 10:1 in the absence (lanes 1–6) or presence (lanes 7–12) of a 2-fold molar excess of EH or 0.2 mM 5,10-CH$_2$-H$_4$PteGlu$_4$ (lanes 13–18) as described under “Experimental Procedures.” The digests were analyzed as described in the legend for Fig. 1. A, ET; B, ET-His$_6$; C, ETΔ7-His$_6$; D, ETT4A-His$_6$; E, ETL4A-His$_6$. The digestion times in minutes are indicated below the lanes, and each intact protein and the fragments derived from ET and ET-His$_6$ are indicated.

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**TABLE II**

Proteolytic fragments of E. coli T-protein

<table>
<thead>
<tr>
<th>T-protein</th>
<th>Fragment</th>
<th>Size (kDa)</th>
<th>N-terminal sequence</th>
<th>Observed mass [M+H$^+$]</th>
<th>Calculated mass [M+H$^+$]</th>
<th>Peptide assignment</th>
</tr>
</thead>
</table>
| ETΔ7-His$_6$ | F1 | 37.0 | MVDFH | ND$^b$ | Met$^{17,His}$ | Met$^{17,His}$
| | F2 | 19.7 | MVDFH | ND | Met$^{17,His}$ | Met$^{17,His}$
| | F3 | 13.6 | ALYSG | ND | Ala$^{22}$Ly$_{203}$/Arg$^{206}$ | Ala$^{22}$Ly$_{203}$/Arg$^{206}$
| | F4 | 7.3 | MVDFH | 6872.78 | 6870.44 | Met$^{17,His}$ |
| ET-His$_6$ | F5 | 39.0 | AQQTP | 39,767.52 | 39,772.13 | Met$^{17,His}$ |
| | F6 | 30.0 | ALYSG | ND | Met$^{17,His}$ |
| | F7 | 22.6 | AATLF | ND | Met$^{17,His}$ |
| | F8 | 20.3 | AATLF | ND | Met$^{17,His}$ |
| | F9 | 16.3 | AATLF | ND | Met$^{17,His}$ |
| | F10 | 12.5 | AATLF | ND | Met$^{17,His}$ |
| | F11 | 9.0 | AATLF | ND | Met$^{17,His}$ |
| | F12 | 7.2 | GVLRN | ND | Met$^{17,His}$ |
| ET | F5$^c$ | 39.0 | AQQTP | 39,771.85 | 39,772.13 | Met$^{17,His}$ |

$^a$ N-terminal sequence was determined up to the fifth residue.
$^b$ Not determined.
$^c$ The C terminus was predicted from the reactivity with anti-His$_6$ tag antibody.
$^d$ The C terminus was predicted from the molecular mass determined by SDS-PAGE.
$^e$ Predicted from the analysis of F4.

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**Fig. 2.** The effects of substrates on the trypsinolysis of E. coli T-proteins. E. coli T-proteins were digested with TPCK-trypsin at an ETs/trypsin (w/w) ratio of 10:1 in the absence (lanes 1–6) or presence (lanes 7–12) of a 2-fold molar excess of EH or 0.2 mM 5,10-CH$_2$-H$_4$PteGlu$_4$ (lanes 13–18) as described under “Experimental Procedures.” The digests were analyzed as described in the legend for Fig. 1. A, ET; B, ET-His$_6$; C, ETΔ7-His$_6$; D, ETT4A-His$_6$; E, ETL4A-His$_6$. The digestion times in minutes are indicated below the lanes, and each intact protein and the fragments derived from ET and ET-His$_6$ are indicated.
fragmentation of the entire ET-His<sub>6</sub> and EH potentiated the event (Fig. 4).

In contrast to trypsin, Lys-C easily digested ET-His<sub>6</sub> at an ET-His<sub>6</sub>/Lys-C (w/w) ratio of 50:1, in which several fragments accumulated as shown in Fig. 5. Edman sequencing of up to 5 residues of these fragments was carried out, and the C-terminal ends were predicted from the molecular masses determined by SDS-PAGE (Table II). The results revealed that at least four lysine residues at positions of 81, 154, 288, and 360 are favorable cleavage sites. Cleavage at Lys<sup>81</sup> and Lys<sup>288</sup> was strongly prohibited in the presence of EH, although that at Lys<sup>360</sup> was again stimulated by the interaction with EH. About half of the EH that was added, i.e. ET-bound EH, remained intact in contrast with the immediate degradation of free EH.

DISCUSSION

In this study, we used limited proteolysis and mutation analysis to further elucidate the structural nature of ET in the catalytic reaction as well as the function of its N-terminal region in the interaction with EH. The mutant proteins were constructed by replacing two invariant residues among the 7 N-terminal residues, Thr<sup>4</sup> and Leu<sup>6</sup>, with alanine as well as the deletion of up to 7 residues. The introduction of a His<sub>6</sub> tag to the C-terminal end not only facilitated the purification of proteins having activity comparable with that of proteins without His<sub>6</sub> but also provided a convenient tool for detecting local conformational change at the C terminus, as mentioned below. A single mutation, T<sup>4</sup>A or L<sup>6</sup>A, resulted in a 4- or 5-fold decrease in the <i>k<sub>&text</sub>/K<sub>m</sub></i> value for reduced EH, respectively, compared with ET, indicating the importance of these residues in the T-protein activity.

The purified wild type and mutant proteins were subjected to limited proteolysis. The difference in the profile of digestion of ET-His<sub>6</sub> and ET<sup>Δ7</sup>-His<sub>6</sub> by a small amount of TPCK-trypsin demonstrated that Arg<sup>16</sup> in ET<sup>Δ7</sup>-His<sub>6</sub> is oriented toward a trypsin-accessible location. After cleavage at Arg<sup>16</sup> and loss of 16 N-terminal residues, the remaining C-terminal polypeptide chain would be unfolded and attacked by trypsin. Because EH and folate substrate substantially prevented the latter event, it is conceivable that they facilitate the intramolecular interaction between the relatively stable N-terminal two-thirds and the extended C-terminal one-third of ET. These findings are consistent with our previous suggestion that Lys<sup>352</sup> serves as the binding site for the first glutamate residue nearest the p-aminobenzoic acid ring of 5,10-CH<sub>2</sub>-H<sub>4</sub>PteGlu<sub>4</sub> and that Lys<sup>81</sup> and Lys<sup>78</sup> participate in the binding with the second and third glutamate residue, respectively (11). Consequently, the
N-terminal region might serve as the molecular hasp locking its own molecule in a compact fold. The point mutations, L6A and T4A, also resulted in an extended N terminus, probably because of the loss of interaction between these residues and some others. The contribution of Leu6 is likely to be essential to the event. Furthermore, the marked reduction (165-fold) in the $k_{cat}/K_m$ value of ET-$\Delta^7$His$_6$ for reduced EH and the absence of cross-linking between Lys$^{288}$ of ET-$\Delta^7$ and Asp$^{34}$ of EH (12) may reflect the disordered conformation in the stabilized C-terminal polypeptide. In this connection, it is worth noting that two point mutations in the N-terminal region of human T-protein, H42R (19) and G47R (20), cause the clinical disorder nonketotic hyperglycinemia. His$^{42}$ and Gly$^{47}$ of the human precursor T-protein correspond to His$^{10}$ and Ala$^{15}$, respectively, of ET. His$^{42}$ is conserved in all eight T-proteins reported thus far, and Gly$^{47}$ is present in all but E. coli T-protein (11).

Extensive sequencing of the Lys-C digests of ET-His$_6$ demonstrated 4 sensitive lysine residues, Lys$^{81}$, Lys$^{154}$, Lys$^{288}$, and Lys$^{360}$. It is well known that residues sensitive to proteolytic cleavage are localized to regions that are solvent-accessible, unstructured, or flexible. The results are quite reasonable because functional lysine residues such as Lys$^{81}$ and Lys$^{288}$ are situated in a solvent-accessible location. Lys$^{288}$ might be masked by the interaction with Asp$^{34}$ of EH. The significant protection by EH of the cleavage at Lys$^{81}$ indicates a buried cross-linking between Lys$^{288}$ of ET and the substrate and ammonia (3). In the study with ET, we could not detect the small peptide that results in the cleavage at Lys$^{81}$ by SDS-PAGE nor detect the small peptide that was released, Ala$^{361}$-Val-Ala$^{363}$. However, detection of polypeptide Ala$^1$-Lys$^{360}$ in the tryptic digest of ET in the presence of EH ruled out the possibility that the positioning of Lys$^{288}$ near the surface of the ET-EH complex may be due to the addition of a His$_6$ tag at the C terminus.

The orientation of folate in the cavity is compatible with the prevalent model revealed Ordered Ter Bi mechanism of the reverse reaction catalyzed by T-protein, in which reduced EH is the first substrate that binds ET followed by the folate substrate binding site, and other related regions. These results are again consistent with our conclusions. Guilhauits et al. (22) proposed that the role of T-protein is not only to locate the tetrahydrofolate cofactor in a position favorable for a nucleophilic attack on the methylene carbon but also to destabilize the T-protein in order to facilitate the unlocking of the arm and initiate the reaction. Its structural nature might be elucidated by crystallographic analysis, a project that is now in progress.

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