Isolation of a Covalent Steady-state Intermediate in Glutamylate 60 Mutants of Thymidylate Synthase*

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Glutamylate 60 of thymidylate synthase coordinates a hydrogen bond network important in proton transfer reactions to and from the substrate dUMP. The E60A and E60L mutants of Lactobacillus casei thymidylate synthase catalyzed tritium exchange from [5-3H]dUMP for solvent protons faster than dTMP formation, indicating accumulation of a steady-state intermediate and a change in partitioning of the intermediate. A covalent complex consisting of E60A or E60L thymidylate synthase, dUMP, and the cofactor CH$_2$H$_4$folate was isolated on SDS-polyacrylamide gel electrophoresis and shown to be chemically and kinetically competent to form dTMP. These results provide proof of the formation of a covalent steady-state intermediate in the reaction pathway of thymidylate synthase and demonstrate that the rate-determining step in the mutants occurs during conversion of the covalent intermediate to dTMP.

Thymidylate synthase (TS,
EC 2.1.1.45) catalyzes the reductive methylation of dUMP by CH$_2$H$_4$folate to give dTMP and H$_2$folate. In recent years, much insight has been gained about the structure and mechanism of this enzyme. Sequences from over 20 sources have revealed that TS is among the most conserved of known enzymes (1, 2), and x-ray crystal structures of several free and bound forms of the enzyme have pointed to key residues involved in substrate binding and catalysis (3–6). We have been particularly interested in correlations of structure-function relationships of TS as probed by the consequences of mutagenesis. In the present work, we show that Glu-60 mutants of TS catalyze formation of an isolatable, covalent steady-state intermediate. This represents one of the few reports in which a mutation of an enzyme results in accumulation and isolation of a stable, normal steady-state intermediate (7, 8). The currently accepted minimal mechanism of TS is depicted in Scheme I. After Michael addition of Cys-198, to which a mutation of an enzyme results in accumulation and a steady-state intermediate; here, Cys-198 of the enzyme is covalently attached to C-6 of dUMP and the one-carbon unit of the cofactor to C-5 of dUMP. Intermediate III is directly analogous to the much studied ternary complex formed between TS, dUMP, and CH$_2$H$_4$folate (9, 10). In the reaction pathway leading to dTMP, it is proposed that the H-5 of intermediate III is removed as a proton, followed by β-elimination of H$_2$folate and hydride transfer to give the products, dTMP and H$_2$folate. As indicated, covalent bond changes are believed to be facilitated by water-mediated general acid-base-catalyzed proton transfers at O-4 and C-5 of the heterocycle (9). A complex containing TS, dUMP, and CH$_2$H$_4$folate, which is presumed to be III, is isolatable by rapid acid quenching of ongoing TS reactions (11); however, the putative intermediate is formed and processed too rapidly to allow convenient study, and we were unable to isolate it on SDS-PAGE.

Crystallographic studies have shown that the completely conserved Glu-60 of TS is involved in an extensive hydrogen bond network that includes several conserved side chains of the enzyme, ordered water molecules, and the pyrimidine ring of the substrate (Fig. 1) (5, 12). Mutation of Glu-60 leads to large losses in catalytic activity (13, 14), and it has been proposed that this residue plays a role in stabilizing the incipient negative charge at O-4 of dUMP (12) or aids in the opening of the imidazolidinone ring of CH$_2$H$_4$folate (4). We show here that mutation of Glu-60 to Ala or Leu affects the partitioning of the putative steady-state intermediate III and thereby alters its putative formation.

MATERIALS AND METHODS

Mutagenesis and Protein Purification.—The E60A and E60L mutants were constructed by cassette mutagenesis of the L. casei TS synthetic gene in plasmid pSCTS13 (15). The 56-base pair Bci/ParI fragment of pSCTS13 was replaced by a "stuffer" oligonucleotide, 5'-GATCAGGGCCGC-AGGCCTGACAGGTATACCAATTTCTCTCTCTCTCTGACA-3'.

To obtain pSCTS13-stuffer. The stuffer was obtained using a 58-nucleotide self-priming oligonucleotide that was filled using T4 DNA polymerase and digested by BciI and ParI to generate cohesive ends (16). The Stul/PstI component of the "stuffer" retains the wild-type TS sequence; the BciI/Stul component does not encode TS and has an unique NotI site for restriction purification (17). The unique Stul site was introduced into pSCTS13-stuffer to reduce the size of the synthetic oligonucleotide cassette needed for mutagenesis from 56 base pairs (BciI/ParI) in the original synthetic TS gene to 32 base pairs (BciI/Stul). A mutagenic oligonucleotide duplex cassette containing 5'-GATCAGGGCCGC for CTGCTGCTGTTT-3' was inserted into the BciI and Stul sites of pSCTS13-stuffer; the bases underlined change codon 60 from Glu to Ala or Leu. The mutant enzymes were purified to homogeneity as described previously (18).

Enzyme Assays—TS activity was monitored spectrophotometrically at 340 nm (19). The standard assay buffer for dTMP formation contained 50 mM TES, pH 7.4, 25 mM MgCl$_2$, 6.5 mM formaldehyde, 1 mM EDTA, and 75 mM β-mercaptoethanol. TS activity of E60A or E60L mutants, which was too low to be measured spectrophotometrically, was monitored by HPLC analysis. HPLC was performed using a Rainin HPLC equipped with a Hewlett-Packard 1040A diode array detector (20). Isocratic separation of dUMP and dTMP was accomplished on an Altusphere IP column using 5 mM KH$_2$PO$_4$, pH 7.0, 5 mM tetra-n-butylammonium sulfate, and 2.5% (v/v) acetonitrile as the eluant with a flow rate of 1 mL/min. Retention volumes for dUMP and dTMP.
were 15 and 26 ml, respectively. For analysis of radioactive reactants and products, dUMP and dTMP UV markers were added to samples prior to chromatography.

TS-catalyzed tritium release from [5-3H]dUMP was monitored by the decrease in the H/C ratio of [2-14C,5-3H]dUMP (21). Reaction mixtures contained 0.5–3 μM TS, 200 μM [2-14C,5-3H]dUMP (20 μCi of H/C mmol, 4 mM of 14C/mmol), and 400 μM [6-3H]CH4 folate in standard TES assay buffer at 25 °C. Aliquots (20 μl) were assayed for tritium release as described (20). The exchange of tritium from [5-3H]dUMP for solvent protons was monitored by analysis of the H/C ratio of [2-14C,5-3H]dUMP isolated by HPLC.

SDS-PAGE of the Covalent Intermediate—Covalent ternary complexes were formed by incubating a mixture containing (a) 4.5 μM E60A or E60L TS, 6.7 μM [6-3H]dUMP (15 Ci/mmol), and 400 μM CH4 folate or (b) 4.5 μM E60A or E60L TS, 400 μM dUMP, and 560 μM [6-3H]CH4 folate (26.6 Ci/mmol in standard TES assay buffer at 25 °C. Aliquots (15 μl) were denatured at various times and analyzed on 12% SDS-PAGE as described (20).

For quantitation and assessment of kinetic parameters of the E60A or E60L TS-[6-3H]dUMP-CH4 folate covalent complex, the radioactivity associated with the protein band at its optimum formation was compared with that of the wild-type TS-[6-3H]dUMP-CH4 folate covalent complex, which contains one FdUMP molecule/TS monomer. Coomassie-stained protein bands were excised, solubilized using Solvable (DuPont NEN), and counted in 10 ml of Aquasol-2 (DuPont NEN). The reaction mixtures contained 4.5 μM E60A or E60L TS, 200 μM [6-3H]dUMP (0.5 Ci/mmol), and 400 μM CH4 folate in standard TES assay buffer at 25 °C. The rate constant for disappearance of the covalent complex was obtained by adding a 100-fold excess of non-radioactive dUMP after the maximum formation of the complex and then monitoring the first order rate decrease of the radioactivity associated with the complex. The apparent first order rate constant for formation of the covalent complex was calculated by dividing the initial rate of formation by the concentration of TS monomer.

RESULTS AND DISCUSSION

The TS reaction can be monitored by following either dTMP formation or the release of tritium from [5-3H]dUMP, which accompanies dTMP formation. With wild-type TS, the observed rates of these reactions are essentially identical (Table I), indicating that tritium release occurs concomitantly with methylation. With E60L and E60A TS, there was a retardation of both the CH4 folate-dependent tritium release from [5-3H]dUMP (600-1,400-fold) and dTMP formation (25,000-fold). Importantly, with the E60A and E60L mutants, the CH4 folate-dependent tritium release from [5-3H]dUMP was 20- and 40-fold faster, respectively, than the dTMP formation. In the absence of cofactor, tritium release was negligible (<0.02 min⁻¹). The uncoupling of 5-tritium release from 5-methylation indicates a change in the partitioning of an intermediate in the reaction pathway.

The rapid release of tritium from [5-3H]dUMP compared with dTMP formation by the Glu-60 mutants can only be explained by an exchange reaction, where the tritium of the substrate is replaced by solvent protons faster than by one-carbon units of the cofactor. Indeed, exchange of tritium from [5-3H]dUMP was directly demonstrated by analysis of the HPLC-isolated substrate during the course of the reaction. From the reaction mechanism (Scheme I) it can be seen that the exchange likely occurs from (a) proton abstraction from III to give IV, followed by (b) re-protonation with water, and (c) reversal of intermediate III to yield dUMP. The tritium released from III must equilibrate with solvent protons in order to observe the exchange; if this were slow, the observed rate of tritium exchange would be a low estimate of the net rate of formation of III. In itself, the cofactor-dependent exchange reaction provides evidence for the putative intermediate III.

The observation that the E60A and E60L TS-catalyzed tritium exchange is faster than methylation indicates that the rate-determining step of dTMP formation is subsequent to formation of putative intermediate III. This suggested to us that III may accumulate. A mixture containing E60L TS, [6-3H]dUMP, and CH4 folate was incubated, denatured at various times, and subjected to SDS-PAGE. As shown in Fig.
When CH₂H₄folate was omitted, no radioactive protein band was observed. The protein-bound radioactivity reached a maximum of 2% of TS monomers at about 10 min and then slowly disappeared (Fig. 2B). As estimated by SDS-PAGE, the apparent first order rate constant for formation of the ternary complex was 1.4 min⁻¹, and the rate constant for its disappearance was 0.07 min⁻¹. After all radioactivity was lost from the covalent complex, HPLC analysis of the reaction mixture demonstrated that the radioactive dUMP was completely converted to dTMP. When [6-³²P]dUMP, a similar radioactive protein band was formed (data not shown). The results for E60A mutant were essentially the same as that of the E60L mutant except that the rate constants for the covalent complex of E60A mutant were not measured.

Thus, E60A and E60L TS form stable covalent ternary complexes, which contain both dUMP and CH₂H₄folate. The observation that the complex was converted to dTMP shows that it is chemically competent as an intermediate in the TS reaction. The rate constant for the disappearance of III in the E60L mutant (0.07 min⁻¹) is in accord with kcat for dTMP synthesis (0.03 min⁻¹) under steady-state conditions. Thus, the covalent complex is kinetically competent as an intermediate in dTMP formation. In the accepted mechanism of TS (Scheme I), III is the only candidate for a stable, covalent ternary complex that could fulfill these criteria for a covalent intermediate.

The present results can be correlated with the structure of TS to provide insight into the specific role of Glu-60 in catalysis. Mutation of Glu-60 would modify either directly or through a hydrogen-bonded water molecule, and retardation of dTMP formation. In the transition state, conversion of IV to V also requires proton transfers at O-4, which would likewise be effected by mutation of Glu-60, and this might lead to further retardation of dTMP formation and the observed accumulation of III. Further, since Glu-60 is coordinated through water molecules to both O-4 of dUMP and N-10 of CH₂H₄folate, it is possible that it plays a role in maintaining proper orientations of the activated pyrimidine ring and cofactor (V) for hydride transfer.

In summary, taken together, the experiments described here provide proof of the formation of a covalent intermediate in the TS reaction, localize the rate-determining step of the reaction, and provide insight for the molecular role of Glu-60. First, the CH₂H₄folate-dependent TS Glu-60 mutant-catalyzed exchange of tritium from [5-³²P]dUMP for solvent protons provides evidence for an intermediate with an exchangeable proton; the expected properties of III make it the likely candidate for this intermediate. Second, isolation of a covalent complex containing both dUMP and CH₂H₄folate, which is chemically and kinetically competent to form dTMP, is only consistent with intermediate III. Third, the H-5 exchange reaction and accumulation of the covalent intermediate show that the rate-determining step occurs during conversion of III to dTMP. The results of our experiments meet the basic criteria for demonstration of a covalent intermediate in enzymatic catalysis (22, 23).

Finally, structure-function considerations lead us to suggest that Glu-60 serves a role in coordinating a hydrogen bond network that promotes proton transfer reactions at O-4 of dUMP and N-10 of the cofactor and orienting the pyrimidine ring of substrate and the pterin ring of the cofactor for hydride transfer. The ability to directly observe intermediate III with TS Glu-60 mutants provides a unique opportunity to study the catalytic details of the formation and reaction of a steady-state intermediate in the TS reaction.

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