Probing Electrostatic Channeling in Protozoal Bifunctional Thymidylate Synthase-Dihydrofolate Reductase Using Site-directed Mutagenesis*

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In this study we used site-directed mutagenesis to test the hypothesis that substrate channeling in the bifunctional thymidylate synthase-dihydrofolate reductase enzyme from Leishmania major occurs via electrostatic interactions between the negatively charged dihydrofolate produced at thymidylate synthase and a series of lysine and arginine residues on the surface of the protein. Accordingly, 12 charge reversal or charge neutralization mutants were made, with up to 6 putative channel residues changed at once. The mutants were assessed for impaired channeling using two criteria: a lag in product formation at dihydrofolate reductase and an increase in dihydrofolate accumulation. Surprisingly, none of the mutations produced changes consistent with impaired channeling, so our findings do not support the electrostatic channeling hypothesis. Burst experiments confirmed that the mutants also did not interfere with intermediate formation at thymidylate synthase. One mutant, K282E/R283E, was found to be thymidylate synthase-dead because of an impaired ability to form the covalent enzyme-methylene tetrahydrofolate-deoxyuridylate complex prerequisite for chemical catalysis.

Electrostatic channeling is a mechanism proposed based on the crystal structure of bifunctional thymidylate synthase-dihydrofolate reductase (TS-DHFR) from Leishmania major that would enable negatively charged dihydrofolate produced at the TS active site to be handed off along a series of solvent-exposed lysine and arginine residues to the DHFR active site, where it is converted to tetrahydrofolate (H$_4$folate) (1).

TS and DHFR are crucial enzymes, found in all species. TS represents the only means of de novo synthesis of 2'-deoxythymidylate (dTMP) for DNA synthesis, via reductive methylation of 2'-deoxyuridylate (dUMP) with methylene tetrahydrofolate (CH$_2$H$_4$folate), producing dihydrofolate (H$_2$folate) in the process.

DHFR catalyzes the reduction of H$_2$folate by NADPH to generate H$_4$folate, used for one carbon unit transfer reactions in several biochemical processes, including thymidylate, purine, and amino acid biosynthesis. Only in protozoal parasites and some plants however, are TS and DHFR found on the same polypeptide chain, leading to the hypothesis that in these organisms, H$_2$folate may be channeled from the TS active site to that of DHFR, never equilibrating with bulk solution (Fig. 1A).

When the crystal structure of L. major TS-DHFR was solved (2.9 Å resolution), a 40 Å “electrostatic highway” across the surface of the protein was proposed as an explanation for how channeling may occur (1, 2). We sought to test the electrostatic channeling hypothesis with two parallel approaches. In this report we present results of mutagenesis of solvent-exposed basic residues comprising the electrostatic highway. In an earlier paper, we reported the findings from targeted molecular docking searches to identify small molecule inhibitors that bind in this region, as a means to block the putative channel (3).

There are precedents for channeling among bifunctional enzymes, of which tryptophan synthase from Salmonella typhimurium is one of the best characterized examples (4–10). Tryptophan synthase is an $\alpha$,$\beta$ enzyme complex: the $\alpha$ subunit catalyzes the aldol cleavage of indole-3-glycerol phosphate to indole and glyceraldehyde 3-phosphate, whereas the $\beta$ subunit catalyzes the condensation of indole with serine to form tryptophan. Solution of the crystal structure of tryptophan synthase from S. typhimurium revealed a 25 Å hydrophobic tunnel connecting the active sites (4). There is also kinetic evidence for a conformational change following formation of the aminoaacrylate intermediate at the $\beta$ subunit, which accelerates the rate of catalysis at the $\alpha$ subunit by $\sim$ 150-fold (6). Buildup of indole is only observed in single enzyme turnover of the $\alpha\beta$ reaction when channeling is obstructed by site-directed mutagenesis (7, 8). Although there is no obvious hydrophobic tunnel through L. major TS-DHFR, there is kinetic evidence for substrate channeling, notably the absence of a lag in product formation at DHFR and a lack of buildup of H$_2$folate (11, 12). Also similar to tryptophan synthase, in bifunctional TS-DHFR, the second enzyme (DHFR) is faster than the first (TS), and there is evidence for domain-domain communication (12).

Steady-state kinetics indicates that there is a lag in formation of the products of the DHFR reaction (NADP and H$_4$folate)
when monofunctional TS and DHFR enzymes are combined, but not in the case of bifunctional TS-DHFR from *Leishmania tropica* (11). This suggests that in the case of the bifunctional enzyme only, H\textsubscript{2}folate produced at the TS site is transferred directly to the DHFR site, rather than first equilibrating in bulk solvent. Direct evidence for substrate channeling has been obtained by transient kinetic analysis. It was shown by rapid chemical quench that in single enzyme turnover experiments with *L. major*, there is no lag in H\textsubscript{2}folate production, and little H\textsubscript{2}folate accumulation is observed, again suggesting direct transfer of H\textsubscript{2}folate from TS to DHFR (12).

Prior to this report there was no direct evidence for electrostatic guidance of H\textsubscript{2}folate as the mechanism by which channeling occurs. There are, however, precedents for electrostatic steering in other enzyme systems (13–17), and structural analysis and Brownian dynamics simulation techniques applied to TS-DHFR provide support for the electrostatic channeling hypothesis (1, 18, 19). The crystal structure of *L. major* TS-DHFR revealed that the two active sites are located on the same face of the protein, separated by a distance of 40 Å. Because H\textsubscript{2}folate has a charge of −2 and polyglutamylated folate substrates found in nature are even more highly negatively charged, distribution of positively charged amino acids (lysine and arginine) on the surface of TS-DHFR was examined. This elucidated a highly positively charged electrostatic potential surface forming the solvent-exposed path connecting TS and DHFR, with a generally negatively charged surrounding surface (1). In the TS domain, contributors to the positively charged potential are Lys-282, Arg-283, and Arg-287, conserved in all TS species. DHFR is much less conserved than TS, and in the DHFR domain of the *L. major* bifunctional enzyme there is a 12-amino acid loop not present in *Escherichia coli*; 6 of these residues are positively charged. The positively charged residues, Lys-66, 67, 72, and 73, and Arg-64 and 74, are solvent-exposed and also take part in the putative channel (Fig. 1B). TS is connected to DHFR by a very short tether; it is predicted that torsional rotation of Arg-287 would position its side chain 10–12 Å from that of Lys-73 in the DHFR domain (1). Brownian dynamics simulation also predicts that in the presence of electrostatic effects, 95% of substrate with charge −2 exiting the TS site would reach the DHFR site, whereas only 6% of substrate would channel in the absence of electrostatics (13).

Although the primary goal of this research was to determine whether solvent-exposed basic residues in the shallow groove region of TS-DHFR participate in electrostatic channeling of H\textsubscript{2}folate, an ancillary goal was to use TS pre-steady-state burst experiments to ascertain whether these same residues might mediate domain-domain communication, or conformational changes induced upon ligand binding at one active site affect activity at the active site of the other enzyme. One conformational change that is known to occur involves TS catalysis: following ordered substrate binding (dUMP then CH\textsubscript{2}H\textsubscript{4}folate), the C terminus of TS closes over the substrates to create an active site cavity shielded from solvent (20–25). It is thought that this is followed by the formation of an iminium ion involving the bridge methylene and N4 of CH\textsubscript{2}H\textsubscript{4}folate (25). We showed previously, using the wild-type enzyme, that there is a burst in consumption of the cofactor, CH\textsubscript{2}H\textsubscript{4}folate, at TS (26). Because it is known that chemistry is rate-limiting at TS, the observation of a burst in CH\textsubscript{2}H\textsubscript{4}folate consumption signals the accumulation of a TS intermediate, likely the iminium form of CH\textsubscript{2}H\textsubscript{4}folate (22–24).

If electrostatic guidance, either via diffuse field effects or by an ordered hand-off mechanism, does account for channeling, or if the basic residues are crucial for domain-domain communication, the channel region could serve as a new drug target in parasites. Targeting the channel between the TS and DHFR in bifunctional enzymes has the potential to produce more specific therapies, with fewer side effects than traditional active site-directed medications, for protozoal diseases including malaria and toxoplasmosis. Previously reported results with eosin B signify an important step toward establishing proof of the principle that the putative channel region of TS-DHFR can serve as a molecular target that when inhibited, results in parasite death (3).

In this report we probe the electrostatic channel hypothesis by mutation of solvent-exposed basic residues predicted to participate in channeling. These TS-DHFR channel mutants are assessed for impaired channeling using two criteria: a lag in product formation at DHFR and increased H\textsubscript{2}folate accumulation. In addition, pre-steady-state burst experiments are used to examine effects on TS catalysis, and one interesting mutant is investigated further.

**EXPERIMENTAL PROCEDURES**

*Chemicals—* All buffers and other reagents employed were of the highest chemical purity. Millipore ultrapure water was used for all

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*FIG. 1. Electrostatic channeling by TS-DHFR.* A, schematic: electrostatic channeling directly transfers the TS product, H\textsubscript{2}folate, across the surface of the protein to the DHFR active site; whereas in the absence of channeling, H\textsubscript{2}folate leaves TS, equilibrates with bulk solution, and rebinds at the DHFR site. B, structure of TS-DHFR from *L. major* with TS shown in red, DHFR in blue, TS and DHFR ligands in green, and solvent-exposed lysines and arginines in magenta.
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solutions. \( \text{CH}_2\text{H}_4\text{folate} \) and \( \text{H}_4\text{folate} \) were purchased from Schircks Laboratories (Switzerland). \( \text{H}_2\text{folate} \) was synthesized by reduction of folic acid with sodium borohydride. Trinitium-labeled \( \text{H}_2\text{folate} \) and \( \text{CH}_2\text{H}_4\text{folate} \) were synthesized using tritiated folic acid as a starting material. The \( \text{[3',5'-7,9-3H]folic acid} \) as well as \( \text{[14C]labeled dUMP} \) and \( \text{FdUMP} \) (5-fluorooxuryridine-5'-[\( \text{14C}] \) monophosphate) were obtained from Moravek Biochemicals (Brea, CA). Trinitium-labeled \( \text{H}_2\text{folate} \) was chemically prepared from the reduction of folate by sodium hydrosulfite. TS-28903. Trinitium-labeled \( \text{H}_2\text{folate} \) was converted to trinitiated \( \text{H}_2\text{folate} \) by \textit{L. major} TS-DHFR + NADPH (DHFR reaction) and condensed with formaldehyde to form \( \text{CH}_2\text{H}_4\text{folate} \). The natural (6R)-\( \text{CH}_2\text{H}_4\text{folate} \) enantiomer was purified by DE52 anion exchange chromatography (Whatman Co.) and used as homogenous \( \text{H}_4\text{folate} \). Trinitiated \( \text{H}_2\text{folate} \) and CH2H4folate solutions were stored in argon purged vials at \(-80^\circ \text{C} \). NADPH and dUMP were purchased from Sigma; the concentration of NADPH was determined by analysis of the cell pellet (data not shown). Mutant proteins were mutants. Mutation of Lys-73 or Arg-74 led to very low yields of active protein concentration. TS active site titrations were performed. 1 different numbers of functional active sites at the same protein concentration, TS active site titrations were performed. 1 \( \mu \text{M} \) tritiated \( \text{CH}_2\text{H}_4\text{folate} \) was incubated with 20 \( \mu \text{M} \) FdUMP (5-fluorodeoxyuridine-5'-[\( \text{14C}] \) monophosphate) to form \( \text{CH}_2\text{H}_4\text{folate} \). The natural (6R)-\( \text{CH}_2\text{H}_4\text{folate} \) enantiomer was purified by DE52 anion exchange chromatography (Whatman Co.) and used as homogenous \( \text{H}_4\text{folate} \). Trinitium-labeled \( \text{H}_2\text{folate} \) was converted to trinitiated \( \text{H}_2\text{folate} \) by \textit{L. major} TS-DHFR + NADPH (DHFR reaction) and condensed with formaldehyde to form \( \text{CH}_2\text{H}_4\text{folate} \). The natural (6R)-\( \text{CH}_2\text{H}_4\text{folate} \) enantiomer was purified by DE52 anion exchange chromatography (Whatman Co.) and used as homogenous \( \text{H}_4\text{folate} \). Trinitiated \( \text{H}_2\text{folate} \) and CH2H4folate solutions were stored in argon purged vials at \(-80^\circ \text{C} \). NADPH and dUMP were purchased from Sigma; the concentration of NADPH was determined by analysis of the cell pellet (data not shown). Mutant proteins were mutants. Mutation of Lys-73 or Arg-74 led to very low yields of active enzyme (10-0.5 \( \mu \text{M} \)) and large amounts of insoluble enzyme by gel analysis of the cell pellet (data not shown). Mutant proteins were purified in a manner similar to that for wild-type.


deighted for purification (28). The wild-type protein has both TS and DHFR activities similar to those reported previously (11, 28, 29). Mutations had been added (Fig. 2).

Enzyme—The clone of the wild-type bifunctional TS-DHFR enzyme from \textit{L. major} was a generous gift from C.-C. Kan and D. Matthews, then at Agouron Pharmaceuticals. This clone, harboring the pO2CLSA4-plasmid in an \textit{E. coli} Rue 10 expression vector, was used to obtain protein of high purity (>99%) using methods described previously. Preliminary enzyme activity assays were conducted on small scale and full scale growths prior to purification. Large growths, typically 22 liters, were necessary to obtain sufficient protein from the more impaired mutants. Mutation of Lys-73 or Arg-74 led to very low yields of active enzyme (10-0.5 \( \mu \text{M} \)) and large amounts of insoluble enzyme by gel analysis of the cell pellet (data not shown). Mutant proteins were purified in a manner similar to that for wild-type.

Enzyme Concentrations—The TS-DHFR protein concentration was estimated spectrophotometrically at 280 nm using an extinction coefficient of 67,800 \( \text{M}^{-1}\text{cm}^{-1} \). To rule out the possibility that differences in rates of the wild-type and mutant enzymes were a result of having different numbers of functional active sites at the same protein concentration, TS active site titrations were performed. 1 \( \mu \text{M} \) 10-propargyl-5,8-dideazafolate (PDDF), a folate analog that binds at the TS active site, was titrated with enzyme, and the change in intensity of a fluorescence resonance energy transfer (FRET) peak at 396 nm was recorded on a SLM 4800 fluorometer (Urbana, IL) with the excitation set at 280 nm. Active site titrations were carried out by adding enzyme in small aliquots to minimize any dilution effects and with constant stirring in a 3-ml quartz cuvette at 25 °C. Fluorescence measurements were recorded as an average of four 10-s readings within 15-30 s of enzyme addition, and the recorded fluorescence intensities were corrected for dilution (29). The inflection point occurred when 2 \( \mu \text{M} \) enzyme required to fully bind 1 \( \mu \text{M} \) PDDF, 1 \( \mu \text{M} \) wild-type \textit{L. major} TS-DHFR had been added (Fig. 2).

Enzyme Activity Assays—The DHFR activity was determined by following the decrease in absorbance at 340 nm which accompanies the conversion of substrates NADPH and dUMP to enzyme + NADP and dUMP. The DHFR reaction was monitored by the addition of tritiated CH2H4folate to enzyme + NADP. The TS reaction was also monitored under burst conditions: 100 \( \mu \text{M} \) enzyme + saturating dUMP, mixed with 200 \( \mu \text{M} \) tritiated CH2H4folate. The enzymatic reactions were terminated by quenching with 67 \( \mu \text{M} \) of 0.78 \( \text{N} \) KOH to give a final concentration of 0.54 \( \text{N} \) KOH (12). TS reactions utilizing radionuclabeled dUMP were quenched with 67 \( \mu \text{M} \) of 0.4 \( \text{N} \) HCl. The rate constants for individual single turnover rapid chemical or burst quench experiments were estimated by fitting the data to a single exponential or burst curve using the curve fitting program, Kaleidagraph.

High Performance Liquid Chromatography (HPLC) Analysis—Tritiated products of the rapid quench experiments were quantified by HPLC in combination with a radioactivity flow detector. The HPLC separation was performed using a BDS-Hypersil C18 reverse phase column (250 \( \times \) 4.6 mm, Keystone Scientific, Bellefonte, PA) with a flow rate of 1 \( \text{ml/min} \). An isotric separation using a solvent system of 90% methanol in 180 \( \mu \text{M} \) triethylammonium bicarbonate at pH 8.0 was employed. The elution times were as follows: H2folate, 9 min; H4folate, 18 min; CH2H4folate, 20 min. For separation of dUMP and dTMP, an isotric separation using a solvent system of 200 \( \mu \text{M} \) triethylammonium bicarbonate was used. The elution times were as follows: dUMP, 11 min; dTMP 21.5 min. The HPLC effluent from the column was mixed with liquid scintillation mixture (Monoflow V, National Diagnostics) at a flow rate of 5 \( \text{ml/min} \). Radioactivity was monitored continuously using a Flo-One radioactivity flow detector (Packard Instruments, Downers Grove, IL). The analysis system was automated using a Waters 712B WISP (Milford, MA) autosampler.

Stopped-flow Absorbance/Fluorescence Measurements—Stopped-flow measurements were performed using a Kintek SF-2001 apparatus (Kinetek Instruments) as described previously (12). In the absorbance exper-
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Relative DHFR and TS-DHFR activity, lag in product formation at DHFR, and \( H_2 \) folate accumulation by major TS-DHFR charge reversal and charge neutralization mutants

| Mutant        | DHFR activity | TS-DHFR activity | Lag to DHFR | Increased \( H_2 \) folate |
|---------------|---------------|------------------|------------|---------------------------|
|               | %             | %                | %          | %                         |
| Charge reversal |               |                  |            |                           |
| R283E         | Wild-type     | 40               | No         | No                        |
| R287E         | Wild-type     | 45               | No         | No                        |
| K66E/K67E     | Wild-type     | 0                | No (TS dead) | No (TS dead) |
| K72E          | Wild-type     | Wild-type        | No         | No                        |
| K73E          | Wild-type     | Wild-type        | No         | No                        |
| R64Q/K66Q/K72Q/K73Q | Wild-type | Wild-type | No | No |
| R64Q/K66Q/K67Q/K72Q/R74Q | 65 | 22.5 | No | No |
| Charge neutralization | |                  |            |                           |
| R64Q         | 124           | 144              | No         | No                        |
| R74Q         | 114           | 143              | No         | No                        |
| R64Q/K66Q/K67Q | 143          | 143              | No         | No                        |
| R64Q/K66Q/K67Q/R287Q | Wild-type | 45               | No         | No                        |
| R64Q/K66Q/K67Q/K72Q/K73A/R287Q | 65 | 22.5 | No | No |

Attempts designed to measure the burst in \( CH_2H_4 \) folate consumption in the TS reaction, absorbance at 340 nm was monitored when 25 \( \mu \)M enzyme was preincubated with 1 \( \mu M \) dUMP and buffer (1 \( \mu M \) EDTA, 50 \( \mu M \) MgCl\(_2\), 50 \( \mu M \) Tris, at pH 7.8) and then mixed with 500 \( \mu M \) enzyme, no lag was observed, suggesting that \( H_2 \) folate is channelled directly from TS to DHFR (11). The equation for NADP formation via the DHFR catalyzed oxidation of NADPH and reduction of \( H_2 \) folate produced at TS is

\[
\text{[NADP]} = v_1 (t) + (v_2/v_3)K_m (e^{-v_2t/K_m} - 1) \tag{1}
\]

where \( K_m \) is the \( K_m \) for \( H_2 \) folate, \( v_1 \) is the rate of TS (\( \mu M/min \)) under coupled assay conditions, and \( v_2 \) is the DHFR rate (\( \mu M/min \)) using near saturating substrate concentrations. When the NADP concentration is plotted versus time, the steady-state concentration of \( H_2 \) folate corresponds to \( v_1 / v_3 \). and the predicted lag time prior to steady-state \( H_2 \) folate accumulation in \( K_m / v_3 \).

Spectroscopic signal change from NADPH consumption, monitored at 340 nm, depends on \( H_2 \) folate formation at TS and is thus proportional to TS activity, whereas lag time is inversely proportional to DHFR activity. To amplify signal change and lag time, TS activity must be maximized and DHFR activity minimized. With monofunctional enzymes this can be accomplished by adjusting the relative TS:DHFR ratios. The bifunctional enzyme poses a particular challenge, however, because the ratio of TS to DHFR active sites is fixed at 1:1 (or 0.5:1 if TS half-site activity is taken into account), and the ratio of specific activities is 1:5.7 (TS:DHFR) for \( L. major \) but not \( E. coli \) DHFR (residues 62–73 in \( L. major \)) did not yield soluble enzyme. By doing large growths (typically 22 liters), however, we were still able to make and test 3 combination mutants that included mutation of Lys-73 (Table 1).

**Steady-state Spectroscopic Analysis to Assess NADP Production**

Meek et al. (11) report a lag in production of NADP via the DHFR-catalyzed reduction of \( H_2 \) folate formed by TS in a coupled assay using \( Lactobacillus casei \) monofunctional TS and DHFR. In the case of the \( L. tropica \) bifunctional TS-DHFR enzyme, no lag was observed, suggesting that \( H_2 \) folate is channelled directly from TS to DHFR (11). The equation for NADP formation via the DHFR catalyzed oxidation of NADPH and reduction of \( H_2 \) folate produced at TS is

\[
\text{[NADP]} = v_1 (t) + (v_2/v_3)K_m (e^{-v_2t/K_m} - 1) \tag{1}
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where \( K_m \) is the \( K_m \) for \( H_2 \) folate, \( v_1 \) is the rate of TS (\( \mu M/min \)) under coupled assay conditions, and \( v_2 \) is the DHFR rate (\( \mu M/min \)) using near saturating substrate concentrations. When the NADP concentration is plotted versus time, the steady-state concentration of \( H_2 \) folate corresponds to \( v_1 / v_3 \). and the predicted lag time prior to steady-state \( H_2 \) folate accumulation in \( K_m / v_3 \).

Spectroscopic signal change from NADPH consumption, monitored at 340 nm, depends on \( H_2 \) folate formation at TS and is thus proportional to TS activity, whereas lag time is inversely proportional to DHFR activity. To amplify signal change and lag time, TS activity must be maximized and DHFR activity minimized. With monofunctional enzymes this can be accomplished by adjusting the relative TS:DHFR ratios. The bifunctional enzyme poses a particular challenge, however, because the ratio of TS to DHFR active sites is fixed at 1:1 (or 0.5:1 if TS half-site activity is taken into account), and the ratio of specific activities is 1:5.7 (TS:DHFR) for \( L. major \).

With a \( K_m \) of 0.6 \( \mu M \) for \( H_2 \) folate and the bifunctional enzyme, the steady-state accumulation of \( H_2 \) folate is fixed at 0.11 \( \mu M \), near reported the 0.1 \( \mu M \) detection limit (11), and corresponding to an absorbance change of 6.6 \( \times \) 10\(^{-7} \) AU (\( \epsilon_{340} \) for \( H_2 \) folate and TS-DHFR = 6,000 \( \mu M^{-1} \) \( \mathrm{cm}^{-1} \)). Using a standard (1-cm path length) quartz cuvette, we observed unacceptable signal to noise in both absorbance (340 nm) and fluorescence (ex 340 nm; em 450 nm) spectra, so a 10-cm path length quartz observation cell was employed with a PerkinElmer Lambda 2 UV-visible spectrophotometer. Here 10 \( \mu M \) \( L. major \) TS-DHFR

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**RESULTS**

**Creation of Channel Mutants**—The crystal structure of bifunctional TS-DHFR from \( L. major \) revealed a series of solvent-exposed basic residues between the two active sites, residues hypothesized to be responsible for channeling of negatively charged \( H_2 \) folate from TS to DHFR. We sought to test this hypothesis by mutating lysines and arginines thought to make up the channel to either glutamic acid (charge reversal) or alanine or glutamine (charge neutralization). The residues mutated were Lys-282, Arg-283, and Arg-287 in the TS domain and Lys-66, 67, 72 and 73, and Arg-64 and 74 in the DHFR domain. A total of 12 channel mutants were successfully created with up to 6 residues changed at once; 2 mutants contained changes in both the TS and DHFR domains (Table 1). Consistently, mutation of Lys-73 or Arg-74 led to very low yields of active enzyme and large amounts of insoluble enzyme, suggesting that these DHFR residues may be important for protein folding/domain stability. Insufficient active enzyme for transient kinetics analysis was recovered from attempts to make the following 3 mutants: R74E, K66E/K67E/K72E/K73E/R64Q/R74Q, and R64Q/K66A/K67A/K72E/K73E. Similarly, attempts to completely remove the 12-amino acid basic loop present in \( L. major \) but not \( E. coli \) DHFR (residues 62–73 in \( L. major \)) did not yield soluble enzyme. By doing large growths (typically 22 liters), however, we were still able to make and test 3 combination mutants that included mutation of Lys-73 (Table 1).
represents the lower limit of ability to detect clearly signal change from NADPH consumption following H₂folate production at TS. 10 nM TS-DHFR corresponds to a lag time of 18 s, also near the lower limit of detection when start and mixing times are accounted for.

Using these conditions we observed a lag with *E. coli* mono-functional TS/DHFR but not with wild-type *L. major* TS/DHFR, although one was predicted, supporting the existence of channeling by the bifunctional enzyme (Fig. 3, A and B). The following *L. major* TS-DHFR mutants were tested under similar conditions: R283E, R287E, R64Q, R74Q, and R64Q/K66A/K67A/R287Q. No convincing evidence of a lag in NADP production was observed with any of these putative channel mutants (Fig. 3, C and D).

**Single Enzyme Turnover Experiments to Look for a Lag in H₄Folate Production and Increased H₂Folate Accumulation**—Although steady-state kinetic analysis is an indirect method from which one can infer information about the rate-limiting step of an enzymatic reaction, transient kinetics allows one to measure directly individual steps in a kinetic pathway as well as to define the reaction kinetics of intermediate formation. Transient kinetics has several advantages for investigation of substrate channeling because, in principle, this technique enables one to monitor directly chemical catalysis at each active site as well as the transit of the putative intermediate from one active site to another (10, 30). In the case of bifunctional TS-DHFR, transient kinetic analysis was used to look for a lag in H₄folate production and increased buildup of H₂folate as evidence of impaired channeling.

Single enzyme turnover experiments, which measure the rate of the chemical conversion of substrate to product at the active site under conditions where enzyme concentration is sufficiently high that substrate binding is not rate-limiting, were performed using a rapid chemical quench apparatus. In the case of combination of the *E. coli* monofunctional TS and DHFR, H₂folate concentration rises more rapidly than H₄folate, and there is almost no H₄folate present at the earliest time points, suggesting a lag in product formation at DHFR (Fig. 4, A and B). Early in the time course, H₂folate makes up 44–60% of the tritiated material when equal concentrations of monofunctional *E. coli* TS and DHFR are combined. Conversely, H₄folate is formed from the earliest time points with the wild-type bifunctional TS-DHFR enzyme (*no lag*) and only a modest amount of H₂folate accumulates, peaking at 14% of tritiated material (Fig. 4, C and D). Note that the *E. coli* enzymes are faster than *L. major* TS and DHFR, so overall product formation over the course of 0.1 s is greater.

The putative channel mutants were each evaluated for changes in the rate of the TS-DHFR and DHFR reactions. With each mutant tested, full time courses for both wild-type *L. major* and the mutant were completed, along with t = 0 and t = 60 s controls. The TS reaction was not evaluated independently because the rate of chemistry for TS is significantly slower than that of DHFR in *L. major* (2 s⁻¹ versus 20 s⁻¹), the rate of TS limits and is equivalent to that of TS-DHFR. To monitor the TS-DHFR reaction, the bifunctional TS-DHFR enzyme (50 μM) was preincubated with saturating concentrations of dUMP and NADPH (500 μM each) and then mixed with a limiting amount of radiolabeled CH₂H₄folate (12.5 μM). To monitor the DHFR reaction, the bifunctional TS-DHFR enzyme (50 μM) was preincubated with a saturating concentration of

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**FIG. 3.** Steady-state spectroscopic analysis to assess NADP production by *E. coli* TS + DHFR, *L. major* TS-DHFR, and the *L. major* R64Q, K66A, K67A, and R283E mutants. A, a lag in NADP production is observed in the coupled assay with *E. coli* TS + DHFR at concentrations matching the TS and DHFR activities of 10 nM *L. major* TS-DHFR. No lag was observed with 10 nM *L. major* TS-DHFR, however, supporting the existence of substrate channeling by the bifunctional enzyme (B). A lag was also present in the case of the

[L. major mutants, R64Q, K66A, K67A, at 10 nM (C) and R283E, tested at 20 nM, because TS-DHFR activity is roughly half that of the wild-type enzyme (D).]
NADPH (500 μM) and then mixed with a limiting amount of radiolabeled H₂folate (12.5 μM).

The data in Table I are presented as the ratio of the rate constant obtained for the mutant enzyme divided by that of wild-type; a difference of less than 10% is considered to be like wild-type. The TS mutants R283E and R287E were both found to have approximately half of wild-type TS-DHFR activity, with no impairment of DHFR alone; K282E/R283E was found to be TS-dead, again with no impairment of DHFR. DHFR mutants including R64Q tended to have a slightly faster TS-DHFR rate and a significantly faster DHFR rate. No differences in behavior were observed between charge reversal and charge neutralization mutants.

Representative TS-DHFR and DHFR time courses from the R64Q/K66A/K67A/R287Q mutant are presented in Fig. 5. The R64Q/K66A/K67A/R287Q mutant is slower overall, so at the earliest time points both products (H₂folate and H₄folate) are below detectable limits, but H₂folate is visualized as soon as product is detected (no lag) (Fig. 5B). Although peaking later in the slower, mutant enzyme, the amplitude of maximal H₂folate accumulation is the same for the mutant and wild-type TS-DHFR enzymes. Surprisingly, no lag in H₂folate

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**Fig. 4.** TS-DHFR single turnover reaction time courses from *E. coli* and *L. major*. A and B, TS and DHFR reactions with monofunctional enzymes: 40 μM *E. coli* TS + 40 μM *E. coli* DHFR reacted with 12.5 μM tritiated CH₂H₄folate. CH₂H₄folate consumption (■), as well as H₂folate and H₄folate production are reported (▲ and ●, respectively); note maximal H₂folate accumulation of 45% of tritiated material (A). B, magnification of the early time course illustrating that in the case of *E. coli* TS + DHFR, there is a lag in product formation at DHFR. H₂folate (▲) accumulates before significant conversion to H₄folate (●). C and D compare with the *L. major* TS-DHFR reaction, where when 50 μM bifunctional enzyme is reacted with 12.5 μM tritiated CH₂H₄folate, maximum accumulation of H₂folate is 14% of tritiated product (▼) (C), and H₄folate (●) is formed from the earliest time points (D).
production or buildup of H$\textsubscript{2}$folate beyond that observed with wild-type L. major was seen with any of the mutants (Table I).

Pre-steady-state Burst Experiments to Examine Effect of Mutations on TS Catalysis—Stopped-flow absorbance and rapid chemical quench experiments were performed under pre-steady-state burst conditions (substrate in excess over enzyme) to determine whether the channel residues might participate in conformational change, specifically the domain movement involved in TS catalysis. It is known that chemistry is overall rate-limiting at TS, as demonstrated in Fig. 6A; under pre-steady-state burst conditions, where 25 $\mu$M L. major TS-DHFR is preincubated with excess 90 $\mu$M [$^{14}$C]dUMP prior to mixing with a large excess of 250 $\mu$M CH$_2$H$_4$folate, the TS reaction occurs at a linear steady-state rate with no burst in [$^{14}$C]dUMP consumption or [$^{14}$C]dTMP formation. The absence of a burst confirms that chemistry or a preceding step is rate-limiting in the TS reaction. A pre-steady-state burst is observed for consumption of the cofactor, CH$_2$H$_4$folate; however, signaling formation of a TS intermediate, likely the iminium form of CH$_2$H$_4$folate, for which a step following chemistry (e.g. product release) is rate-limiting (Fig. 6B).

Several of the channel mutants were tested for a burst in CH$_2$H$_4$folate consumption to examine the effect of mutation on TS catalysis. R64Q, R283E, and R287E were tested by rapid chemical quench. R64Q, R64Q/K66A/K67A, R74Q, R283E, and R287E were tested using stopped-flow absorbance. In the case of each of the mutants tested using either technique, a biphasic

![Figure 5](http://www.jbc.org/Downloadedfrom)
Further Characterization of the TS-dead Mutant, K282E/R283E—We sought to determine the molecular mechanism by which the K282E/R283E mutant, with two nonactive site mutations near the folate binding site in TS, is devoid of TS activity. The mutation homologous to K282E in E. coli, K48E, was also found to be TS-dead, but no analysis has been reported (31). As mentioned above, by fluorometry, the concentration of TS active sites capable of binding PDDF with the TS-dead mutant is equivalent to the protein concentration. We examine whether the lack of activity is because of (a) decreased binding affinity for folates, (b) impaired nucleotide binding or inability to form the FdUMP-CH$_2$H$_4$folate-enzyme ternary complex prior to chemistry, or (c) global structural instability.

Binding Affinity for PDDF and CH$_2$H$_4$Folate—Stopped-flow fluorescence was used to measure the binding affinity of K282E/R283E for the folate-analog, PDDF, relative to that of wild-type. The $k_{\text{on}}$ was obtained by titrating 100 nM wild-type or mutant TS-DHFR with increasing amounts of PDDF and plotting $k_{\text{obs}}$ (s$^{-1}$) versus concentration of PDDF added: $k_{\text{obs}} = k_{\text{on}}[\text{PDDF}] + k_{\text{off}}$ (Fig. 7). An independent and more precise way to measure the $k_{\text{off}}$ for PDDF is to measure the rate when PDDF is competed with an excess of a ligand that binds at the same site. This was accomplished by competing 5 μM PDDF with 100 μM CH$_2$H$_4$folate in the presence of 200 nM wild-type or TS-dead L. major TS-DHFR. For the wild-type enzyme, $k_{\text{off}}$ was found to be 2.6 s$^{-1}$, and $k_{\text{on}}$ was 21.3 s$^{-1}$ μM$^{-1}$; $K_d = k_{\text{off}}/k_{\text{on}} = 122$ nM. For the TS-dead mutant, $k_{\text{off}}$ was found to be 2.05 s$^{-1}$, and $k_{\text{on}}$ was 16.52 s$^{-1}$ μM$^{-1}$; so the $K_d$ for PDDF was 124 nM (Fig. 7). The wild-type and TS-dead enzymes also had a similar $k_{\text{off}}$ for CH$_2$H$_4$folate (200 nM enzyme + 5 μM CH$_2$H$_4$folate, competed with 50 μM PDDF): for wild-type TS-DHFR, the $k_{\text{off}}$ for CH$_2$H$_4$folate was found to be 5.83 s$^{-1}$, and it was 4.1 s$^{-1}$ for the TS-dead mutant.

FdUMP Binding and FdUMP-CH$_2$H$_4$Folate-Enzyme Ternary Complex Formation—Size exclusion columns were used to investigate binding of $^{14}$C-labeled FdUMP to the enzyme because no fluorescence signal was detected upon addition of dUMP or the analog, FdUMP. FdUMP nucleotide binding was found to be similar for the wild-type and TS-dead enzymes. When 20 μM radiolabeled FdUMP was added to either 50 μM wild-type or TS-dead TS-DHFR, a small portion of total counts was recovered in the flow-through, representing enzyme-bound FdUMP. It is thought that the low percent bound is a reflection of the high off rate for FdUMP. When excess cold dUMP (100 μM) was added at the same time as the $^{14}$C-labeled FdUMP and enzyme, significantly fewer counts were found in the flow-through, indicating that the FdUMP is able to be competed off by dUMP.

It is known that prior to chemistry, a dUMP-CH$_2$H$_4$folate-enzyme ternary complex is formed. With the wild-type TS enzyme, FdUMP can be used to trap this ternary complex as a covalent intermediate (12, 20). To assess the ability of the mutant enzyme to form the covalent complex, 50 μM enzyme was preincubated with 100 μM excess unlabeled CH$_2$H$_4$folate and 20 μM radiolabeled FdUMP. In this case, ~60% of the total FdUMP was bound to wild-type enzyme, whereas less than 20% was bound to the TS-dead mutant (Fig. 8). When 100 μM excess unlabeled FdUMP was added to the preincubation mix, ~50% of the total FdUMP added still remained bound to the wild-type enzyme, but only 5% remained bound to the mutant (Fig. 8). These results imply that the K282E/R283E TS-dead mutant is unable to form the covalent dUMP-CH$_2$H$_4$folate-enzyme complex requisite properly for chemistry to take place.

**Fig. 6.** TS pre-steady-state burst experiments. A, rapid chemical quench TS burst experiment where 25 μM L. major TS-DHFR is preincubated with excess 90 μM $^{14}$C-dUMP prior to mixing with a large excess of 250 μM CH$_2$H$_4$folate. B and C, stopped-flow absorbance at 340 nm was monitored when 25 μM L. major TS-DHFR was preincubated with a large excess of 1 mM dUMP and then mixed with 500 mM CH$_2$H$_4$folate. B, wild-type TS-DHFR. C, the R283E TS-DHFR mutant enzyme. TS burst with a fast and a slow phase was observed, suggesting that the mutated residues are not critical to TS catalysis. Burst amplitudes were similar for each of the mutants tested, but R283E exhibited a pre-steady-state burst rate that was roughly half of that observed with the wild-type enzyme (33 μM s$^{-1}$ versus 63 μM s$^{-1}$) (Fig. 6C).
Restoration of Activity to Assess Global Stability—From studies in *E. coli*, it is known that TS, which exists as a dimer in most species, exhibits half-the-sites activity, meaning that at any given time, only one half of the TS dimer is kinetically competent (31, 32). In *E. coli* TS it was shown that the amino acid Arg-126 participates in the catalytic site of the opposite half of the dimer and that a TS dimer in which both Arg-126 residues have been mutated to glutamic acid is TS-dead. When a TS-mutant that is dead because of a mutation outside of the active site is combined with the R126E mutant to form a heterodimer, however, full TS activity is restored. In the heterodimer, one half of the dimer now effectively has two mutations (as Arg-126 contributes to the opposite half), but the TS subunit containing R126E is catalytically active, as a normal Arg-126 has been contributed by the nonactive site mutant.

In *E. coli*, one TS-dead mutant whose activity can be restored by heterodimerization is K48E, homologous to K282E in *L. major* (31). We hypothesized that if Lys-282 and Arg-283 were crucial to global stability of the protein, then dissociating the TS dimer and reassociating the K282E/R283E mutant with an active site mutant may not lead to restoration of TS activity. To test whether activity could be restored to K282E/R283E, the equivalent mutation to *E. coli* R126E was made in *L. major* TS-DHFR, R380E. The R308E homodimer was shown to be TS-dead by a spectroscopic enzyme activity assay and by rapid chemical quench. When 0.41 μM R380E was incubated with 4.1 μM K282E/R283E in the presence of urea to facilitate subunit exchange (1 M urea, 25 mM KP, pH to 7.5), activity was restored.

**DISCUSSION**

As a direct test of the electrostatic channeling hypothesis in *L. major* TS-DHFR, 12 putative channel mutants were created: up to 6 amino acids were mutated at once, and 2 mutants contained changes in both the TS and DHFR domains. Both charge neutralization and charge reversal mutants were made. The mutants were evaluated for impaired channeling using two criteria: a lag in product formation at DHFR and increased H2folate accumulation.

The mutants were first analyzed in a steady-state spectroscopic experiment for a lag in production of NADP via the

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**FIG. 7.** Stopped-flow fluorescence experiment to measure the binding affinity of the K283E/R283E mutant for PDDF. A, representative stopped-flow trace of fluorescence at 380 nm versus time, observed upon mixing K282E/R283E TS-DHFR with 2.5 μM PDDF. B, plot of concentration-dependent rate (k_{obs}) versus PDDF concentration.

**FIG. 8.** Spin column assays to assess FdUMP binding and covalent (FdUMP·CH₂H₄folate·enzyme) complex formation by the TS-dead mutant. Percent ¹⁴C-labeled FdUMP (⁺FdUMP) bound to wild-type (black) or mutant (gray) TS-DHFR is reported for various conditions. 1) When 20 μM radiolabeled FdUMP was added to wild-type or 50 μM TS-dead enzyme, a small proportion of total ¹⁴C counts was recovered in the flow-through, representing enzyme-bound ¹⁴C-labeled FdUMP (12 and 7% of counts in with the wild-type and mutant enzymes, respectively). 2) ¹⁴C-labeled FdUMP may be competed off either enzyme with 100 μM excess cold dUMP. 3) Significantly more ⁺FdUMP remains bound to enzyme in the presence of 100 μM excess CH₂H₄folate, although the mutant does not bind as well as wild-type (~15% versus ~60% of ⁺FdUMP bound). Error bars represent differences observed when the experiment was duplicated. 4) Comparatively less ⁺FdUMP can be competed off wild-type enzyme by cold dUMP in the presence of CH₂H₄folate, suggesting that a covalent complex is forming, as predicted. Again, a difference is observed between the wild-type and mutant enzymes. 80% of ⁺FdUMP remains bound to enzyme in the presence of excess CH₂H₄folate and dUMP, whereas only 33% remains bound to the K282E/R283E mutant.
DHFR-catalyzed reduction of H₂folate produced at TS. Meek et al. (11) observed a lag in the case of a monofunctional TS and DHFR-coupled assay but not with the bifunctional TS-DHFR enzyme, suggesting that in the case of the bifunctional enzyme, H₂folate produced at TS is channeled across the surface of the enzyme from TS to DHFR without equilibration into bulk solvent. We observed similar behavior using wild-type monofunctional and bifunctional enzymes; however, no clear lag was observed with any of the putative electrostatic channel mutants, suggesting that at this level of analysis, these mutations were not interfering with channeling behavior. Because we were close to the limits of detection, however, we were not confident of our ability to detect subtle channeling impairment resulting from mutagenesis using this assay. Single enzyme turnover experiments, which allow for direct monitoring of the active sites, were designed, and radiolabeled substrates were used to enhance sensitivity and ability to quantify H₂folate accumulation. The mutants were analyzed under single enzyme turnover conditions by rapid chemical quench for a lag in H₄folate production and an increased accumulation of H₂folate, evidence that, as a result of disruption of the electrostatic channel, H₂folate is now leaving the surface of the enzyme at TS and rebinding the DHFR site after equilibration with bulk solution. A lag in production of H₄folate and a large accumulation of H₂folate were observed when E. coli monofunctional TS and DHFR were combined at a ratio of 1:1. No lag in H₄folate production was observed in the case of the L. major bifunctional TS-DHFR enzyme, and only a small amount of H₂folate accumulated, 3-fold less than with E. coli TS + DHFR.

It is unclear whether the H₂folate observed with the bifunctional enzyme is formed as a result of full TS catalysis or whether it is a breakdown product of the TS iminium ion intermediate. If it is the product of TS catalysis, it suggests that a small percentage of H₂folate dissociates from the wild-type TS-DHFR enzyme and rebinds at DHFR. A much larger accumulation, comparable with that observed with E. coli TS + DHFR, is predicted in the absence of channeling (12). Therefore, what we would like to stress is the 3-fold difference in H₂folate accumulation between E. coli TS + DHFR and L. major TS-DHFR. If the putative electrostatic channeling mutants were even partially channeling-impaired, this 3-fold difference provides ample latitude to detect subtle changes. The difference in behavior between monofunctional TS + DHFR and bifunctional TS-DHFR is, however, unchanged by mutation of the putative electrostatic channeling residues; none of the putative channel mutants exhibited a lag in H₂folate production or increased H₂folate accumulation. Results from single enzyme turnover experiments confirm findings of the steady-state assay: no evidence of impaired chan-
Mutagenesis Does Not Support TS-DHFR Electrostatic Channeling

channeling was observed with any of the putative electrostatic channeling mutants and monoglutamyl folate substrates. We also performed TS experiments under pre-steady-state burst conditions to determine whether the solvent-exposed basic residues are involved in conformational changes associated with TS catalysis, specifically formation of the TS iminium ion intermediate. In the case of the wild-type enzyme and each of the charge reversal or charge neutralization mutants tested, we observed a burst in consumption of the cofactor, CH2H4folate at TS (26). Because chemistry is overall rate limiting at TS (no burst in dUMP consumption or dTMP formation), the observation of a burst in CH2H4folate consumption signifies the presence of a TS intermediate, most likely the iminium form of CH2H4folate. Our results suggest that the putative channel residues are not critical to iminium ion formation. Paralleling our single enzyme turnover findings, the R283E mutant exhibited a burst rate that was roughly half of that observed with the wild-type enzyme, indicating impairment of an early step in the kinetic mechanism.

Additional findings were that mutation of Lys-73 or Arg-74 resulted in largely insoluble protein, suggesting that these residues may play a role in protein folding and that the R64Q mutation resulted in faster rates for both TS and DHFR. It is possible that Arg-64 is involved in domain-domain communication and that its normal role is to limit the rate of the TS reaction, but this has yet to be investigated.

The K282E/R283E mutant was found to be TS-dead. In E. coli, mutation of the residue homologous to K282E alone resulted in a TS-dead enzyme, but no mechanism of inactivation was reported (31). It was predicted that the K282E/R283E mutation would prohibit folate binding at the TS site based on the crystal structure of L. major TS-DHFR (Fig. 9) and because corresponding residues in L. casei are known to participate in polyglutamyl folate binding (1). Surprisingly, binding of CH2H4folate or the folate analog, PDDF, was not impaired in the TS-dead mutant. Instead, our studies suggest that Lys-282 and/or Arg-283 is required for ternary complex formation (dUMP-CH2H4folate-enzyme) prior to chemistry. Because R283E alone results in 40% TS activity but a normal TS burst, indicating formation of the iminium ion intermediate, it follows that Lys-282 is likely the more critical residue for ternary complex formation. It also appears that there is no global structural disturbance because the TS-dead mutant is able to restore activity to the active site mutant, R380E, when heterodimers are formed. Efforts are currently under way to investigate the structural consequences of this mutation by solving the crystal structure in the presence and absence of ligands.

The site-directed mutagenesis data presented in this study do not support the hypothesis that substrate channeling in the bifunctional TS-DHFR enzyme from L. major occurs via electrostatic interactions between the negatively charged H2folate and a series of lysine and arginine residues on the surface of the protein. It now seems probable that channeling instead occurs in conjunction with domain-domain communication or conformational changes induced by ligand binding at one active site that affect activity at the active site of the other enzyme. We have begun to address the coupling of channeling and communication through investigation of a small molecule inhibitor that binds in the channel region (3). Future research will focus on mechanistic and structural determinants of TS-DHFR domain-domain communication as it relates to substrate channeling with the ultimate goal of developing a non-active site therapy for protozoal infection.

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Probing Electrostatic Channeling in Protozoal Bifunctional Thymidylate Synthase-Dihydrofolate Reductase Using Site-directed Mutagenesis
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