Kinetics and Mechanism of Interaction of 10-Propargyl-5,8-dideazafolate with Thymidylate Synthase

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The interaction of *Lactobacillus casei* thymidylate synthase (TS) with 10-propargyl-5,8-dideazafolate (NPQ) in the presence of 2'-deoxyuridylate (dUMP) has been investigated. After formation of a rapdily reversible dUMP–NPQ–enzyme complex, a slow isomerization occurs to provide a ternary complex that can be isolated on nitrocellulose membranes or by gel filtration. Unusual features of the isolable complex are the slow rate by which it is formed ($t_{1/2}$ = 0.88 h) and the slow rate at which it dissociates ($t_{1/2}$ = 26.5 h). The ternary complexes contain 2 mol of dUMP and 2 mol of NPQ bound per mol of dimeric enzyme. Ultraviolet difference spectra of the dUMP–NPQ–TS complex shows a high wavelength maximum that has been attributed to perturbations of the enzyme and/or ligand chromophores that occur upon binding. Data are presented that suggest that the formation of the isolable ternary complex involves nucleophilic attack by a catalytic thiol group of the enzyme to the 6-position of dUMP. Evidence for this is as follows: first, there is a decrease in the absorbance of the pyrimidine chromophore at 265 nm that occurs at the same rate as the formation of the isolable complex; second, using [6-^{3}H]dUMP there is a large, inverse α-secondary kinetic isotope effect (k_H/k_T = 0.83) upon formation of the complex that is in accord with sp^2 to sp^3 rehybridization of the 6-carbon of the heterocycle. Treatment of the complex with sodium dodecyl sulfate ($NaDodSO_4$) results in the dissociation of both ligands in an unmodified form, which is consistent with proposed structure of the complex. Isolable ternary complexes are also formed when the enzyme is incubated with 5-fluoro-2'-deoxyuridylate (FdUMP) and NPQ. Interestingly, the dissociation of FdUMP from these complexes is biphasic, with one-half of the bound nucleotide dissociating at an exceedingly slow rate ($t_{1/2} \simeq 100$ h). The fmdings are discussed with relationship to the possible use of NPQ as an anticancer agent.

Thymidylate synthase (TS) (EC 2.1.1.45) catalyzes the conversion of dUMP and CH_2-H_4folate to dTMP and H_2folate. Because this enzyme is essential for de novo synthesis of dTMP, and hence DNA synthesis, much effort has been invested in studies of the biochemistry, mechanism, and inhibition of this enzyme (for reviews, see ref 2–4). Thus far, the most successful inhibitors of this enzyme have been 5-substituted dUMPs such as FdUMP and NO_2dUMP, which act as potent mechanism-based inhibitors; these analogues reversibly bind to the enzyme and undergo catalytic events in a manner analogous to the normal reaction, ultimately leading to stable covalent complexes involving the addition of the catalytic thiol of the enzyme to the 6-position of the heterocycle. In general, most analogues of the folate cofactor, CH_2-H_4folate, have not been found to be potent and specific inhibitors of TS. The exception to this appears to be derivatives of 5,8-dideazafolate that compete favorably with the cofactor and, in some cases, provide reasonably specific inhibitors of this enzyme.[5-7] In particular, NPQ has been reported to be an extremely potent inhibitor of TS, competitive with respect to CH_2-H_4folate.[8,9] This analogue has also been shown to cause the in vivo inhibition of TS, with depletion of the dTTP pools and resultant perturbation of other intracellular deoxyribonucleoside triphosphates.[9] In the present work, we describe studies of the interaction of NPQ with TS from *Lactobacillus casei* that demonstrates that, after formation of rapidly reversible dUMP–NPQ–TS complexes, a slow isomerization occurs to provide a ternary complex that can be isolated by common physical methods. We describe the properties of these complexes and suggest how they might have relevance to the use of NPQ as an anticancer agent.

Results

Kinetic Studies. Using the conventional spectrophotometric assay for *L. casei* TS and initiating the reaction with the enzyme, NPQ was a competitive inhibitor with respect to CH_2-H_4folate with K_i = 10 nM. However, after incubation of the enzyme with dUMP and NPQ for 20 min, noncompetitive inhibition kinetics were observed. As shown in Figure 1, incubation of [6-^{3}H]dUMP, NPQ, and TS results in a slow formation of a radioactive complex that could be isolated on nitrocellulose filters and, upon completion of reaction, contained 1.9 mol [6-^{3}H]dUMP/mol of dimeric enzyme. When NPQ was omitted, macromolecular bound radioactivity could not be isolated, demonstrating the involvement of the folate analogue in the complex. The fact that NPQ is a good competitive inhibitor of TS at time periods where the amount of isolable complex is negligible suggests that there is a rapid preequilibrium formation of an initial ternary complex followed by isomerization to one that is isolable on nitrocellulose.

The kinetics of formation of the isolable complex was monitored by nitrocellulose filtration of aliquots from solutions containing 10 nM TS and excess amounts of [6-^{3}H]dUMP and NPQ. Under these conditions, formation of the complex gave linear first-order plots from which apparent rate constants, k_{obsd}, were obtained. Assuming

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(1) Abbreviations used are as follows: TS, thymidylate synthase; NPQ, also called 10-propargyl-5,8-dideazafolate or known as CB3717; CH_2-H_4folate, (6*RS*)-L-5,10-methylenetetrahydrofolic acid; dUMP, 2'-deoxyuridylate; FdUMP, 5-fluoro-2'-deoxyuridylate; TES, *N*-tris[(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; $(n\text{-Bu})_4N^+HSO_4^-$, tetrabutylammonium hydrogen sulfate; RV, retention volume. All other abbreviations are as suggested by IUPAC.

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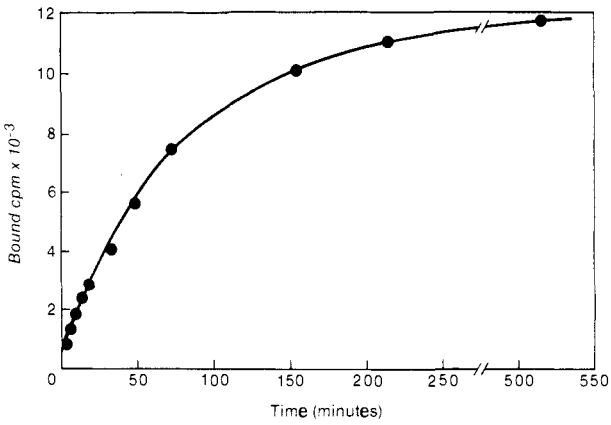

Figure 1. Formation of the [6-^{3}H]dUMP–NPQ–dTMP synthase complex. Enzyme (10 nM) was incubated with 50 μM NPQ for 30 min at 25 °C in the standard buffer. The reaction was initiated by addition of [6-^{3}H]dUMP (18C/mmol) to give a final concentration of 0.25 μM with filter assays performed at the indicated times.

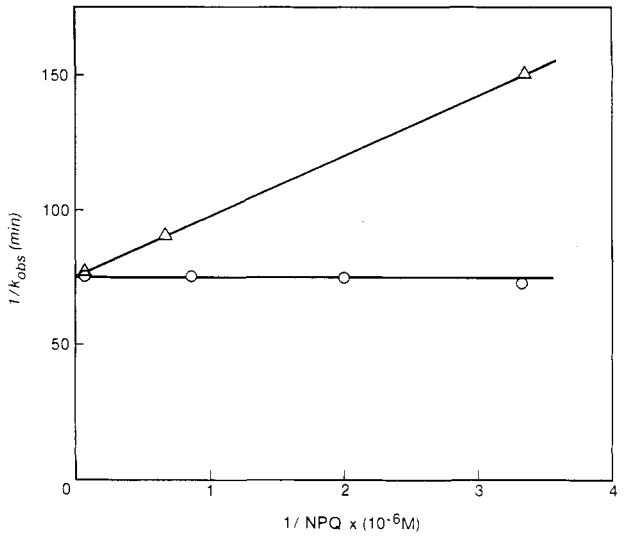

Figure 2. Plot of $1/k_{obsd}$ vs. 1/NPQ with varying [6-^{3}H]dUMP for the formation of the isolable enzyme–dUMP–NPQ complex. [6-^{3}H]dUMP concentrations were (△) 0.25 and (○) 1.1 μM and filter assays were performed as described in the Experimental Section.

that, as in the normal enzyme reaction,[10,11] the interaction is ordered with dUMP binding first and that dissociation of the isolable complex is slow compared to its formation, the formation of the complex may be described by eq 1.

$$\frac{1}{k_{obsd}} = \frac{k_{dUMP}K_{NPQ}}{k_1[dUMP][NPQ]} + \frac{K_{NPQ}}{k_1[NPQ]} + \frac{1}{k_1} \quad (1)$$

Here, K_{dUMP} and K_{NPQ} are the dissociation constants for the ligands in rapidly reversible complexes, and k_1 is the unimolecular rate constant for conversion of the rapidly reversible dUMP–NPQ–TS complex to that which is isolable on nitrocellulose membranes. Figure 2 shows a plot of $1/k_{obsd}$ vs. 1/NPQ at two concentrations of dUMP. At 0.25 μM, k_{obsd} increased with increasing NPQ whereas at 1.1 μM dUMP there was rate saturation. The intercept at the vertical axis gives $k_1 = 0.013$ min^{-1}; with use of this value and $K_{dUMP} \simeq 2.0$ μM,[12,13] K_{NPQ} is calculated from eq 1 to be 40 nM, which is in reasonable agreement with the K_i determined by initial velocity experiments.

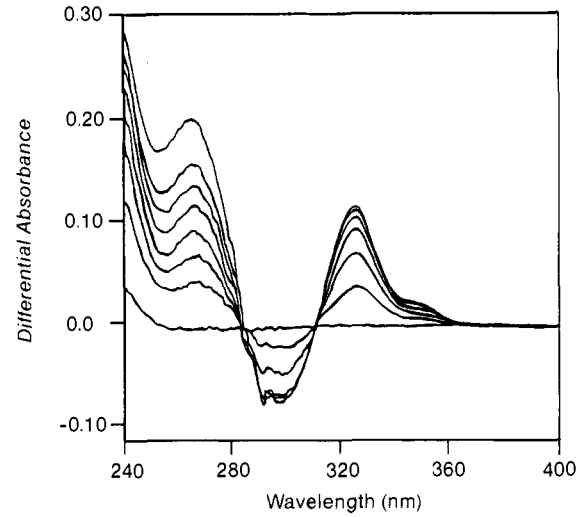

Figure 3. Titration of the dTMP–synthase–NPQ complex with dUMP. The difference spectra shown were obtained by successive 5-μL additions of 0.424 mM dUMP to a sample cuvette containing 36.6 μM NPQ and 6 μL of TS with an equal volume of H_2O added to the reference cuvette which contained only buffer. Each addition corresponds to an absorbance increase at 265 nm and also, prior to saturation, at 325 nm. Stored scans were processed as described in the Experimental Section followed by appropriate subtractions to give the final difference spectra representing the TS–NPQ–dUMP complex vs. TS and NPQ.

The rate of dissociation of [6-^{3}H]dUMP from the ternary complex was determined as follows. The complex was formed with use of 25 nM enzyme, 0.20 μM [6-^{3}H]dUMP, and 50 μM NPQ. After filter binding assays of duplicate 50-μL aliquots, a 200-fold excess of unlabeled dUMP was added, and at intervals aliquots were analyzed by the nitrocellulose filter assay. The dissociation was first order with $t_{1/2} = 26.5$ h.

Similar, albeit less extensive, studies were performed with 5,8-dideazafolate, which is structurally analogous to NPQ except for the lack of the 10-propargyl substituent. With use of 10 nM enzyme, 0.25 μM [6-^{3}H]dUMP, and 50 μM of the folate analogue, the formation of isolable complexes proceeded with $k_{obsd} = 0.50$ min^{-1}, some 38-fold faster than that observed with NPQ under similar conditions. To determine the rate of dissociation, a complex was formed by incubating 10 nM enzyme, 0.25 μM [6-^{3}H]dUMP, and 50 μM 5,8-dideazafoalte for 2 h. After addition of a 200-fold excess of unlabeled dUMP, loss of protein-bound radioactivity was monitored by nitrocellulose filtration of aliquots; dissociation of the complex was first order with $t_{1/2} = 25$ min.

UV Difference Spectroscopy. Figure 3 shows the UV difference spectra obtained upon successive additions of dUMP to a solution containing 6 μM TS and 36 μM NPQ; spectra of identical concentrations of the enzyme and NPQ were subtracted to give the final difference representing the enzyme–NPQ–dUMP complex vs. enzyme and NPQ. The maximum at 325 nm and minimum at 295 nm are characteristic of nucleotide–folate–TS complexes,[2,14] presumably reflecting perturbations of chromophores of NPQ and/or the enzyme. There is also a maximum at 265 nm, the absorbance of which corresponds to the amount of

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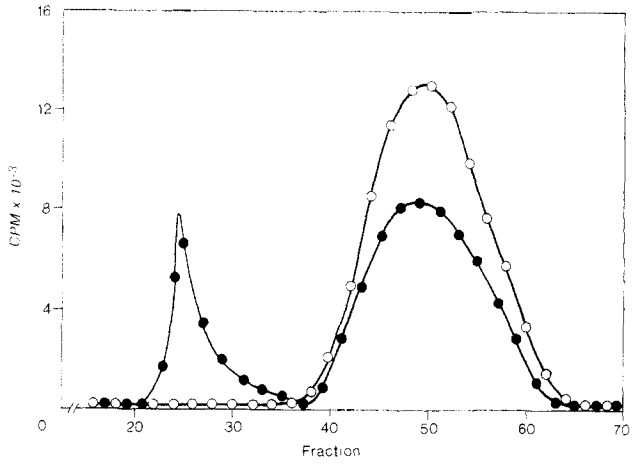

Figure 4. Sephadex G-25 chromatography. The [6-^{3}H]dUMP complex was formed in the standard buffer containing 26 nM enzyme, 50 μM NPQ, and 0.15 μM [6-^{3}H]dUMP. After 18 h a portion (0.070 mL) of the native complex was chromatographed (●) as was an aliquot (0.090 mL) that had previously been treated with 1% $NaDodSO_4$ (2 min, 100 °C) (○).

dUMP present even when the nucleotide is present in limiting concentration and completely bound to the enzyme. As the concentration of dUMP was increased, A_{325} increased linearly until the enzyme was saturated. A plot of ΔA_{325} vs. dUMP/enzyme indicated that 1.9 mol of the nucleotide were bound per mol of enzyme and that $\Delta\epsilon_{325}$ = 19000 M^{-1} cm^{-1}. A similar titration varying NPQ in the presence of 6 μM TS and 50 μM dUMP demonstrated that 1.8 mol of the cofacor analogue was bound per mol of enzyme; in this experiment, $\Delta\epsilon_{325}$ = 18700 M^{-1} cm^{-1} was obtained, which agrees well with that obtained upon titration with dUMP.

When repetitive difference spectra of a solution containing 7 μM TS, 33 μM NPQ, and 17 μM dUMP were taken over a 14-h period, there was no change in the A_{325}. However, there was a first-order decrease in the A_{265} with k_{obsd} = 0.015 min^{-1} which closely corresponds to the value for k_1 obtained from kinetic studies on the formation of the isolable dUMP–NPQ–enzyme complex.

Denaturation of the Ternary Complex. As shown in Figure 4, treatment of the [6-^{3}H]dUMP–NPQ–TS complex with 1% $NaDodSO_4$ resulted in a complete loss of macromolecular-bound radioactivity as monitored by Sephadex G-25 chromatography. The following experiment demonstrated that the bound ligands were released unchanged upon denaturation of the complex. A solution containing 8.0 μM enzyme, 8.0 μM NPQ, and 8.0 μM [6-^{3}H]dUMP was incubated at 25 °C for 18 h; at this time over 90% of the [6-^{3}H]dUMP was bound to the enzyme as determined by the nitrocellulose binding assay. The mixture was treated with 1% $NaDodSO_4$ at 100 °C for 2 min, deproteinized with 2 vol of MeOH, and evaporated to remove the MeOH. HPLC analysis revealed that all of the radioactivity was associated with dUMP and that NPQ was unchanged; overall recoveries were about 90%.

α-Secondary Kinetic Isotope Effects. A solution containing 0.5 μM TS, 50 μM NPQ, and 5.0 μM [2-^{14}C,6-^{3}H]dUMP (54.7 mCi of ^{14}C/mmol; $^3H/^{14}C$ = 6.20) was incubated under standard conditions, and at intervals complexes were isolated on nitrocellulose filters. The $^3H/^{14}C$ ratio of the complexes isolated between 3% and 50% completion of the reaction remained constant at 7.49 ± 0.08 (n = 9). This represents a 21% enrichment of tritium in the complex, which is close to the maximal inverse α-kinetic isotope effect expected for addition of a thiol group to the 6-position of the uracil heterocycle.[15]

A similar experiment was performed utilizing limiting [2-^{14}C,6-^{3}H]dUMP (2 μM; $^3H/^{14}C$ = 6.20) with excess enzyme (5 μM). At early stages of the reaction (10 min) the $^3H/^{14}C$ of the complex was significantly greater than the reactant ($^3H/^{14}C$ = 7.44) and approached that of the reactant as all of the dUMP was bound to the enzyme. In this experiment k_T/k_H was calculated to be 1.20 by using the equation described by Melander.[16]

No isotope effect was observed upon dissociation of [2-^{14}C,6-^{3}H]dUMP from the preformed complex. The complex was formed by incubation of 1.0 μM TS, 50 μM NPQ, and 5.0 μM [2-^{14}C,6-^{3}H]dUMP in the standard buffer at 25 °C for 18 h. A 200-fold excess of unlabeled dUMP was added, and aliquots were assayed by adsorption to nitrocellulose membranes over a period of 96 h (3.6 half-lives). Dissociation of the radioactive ligand was first order over this period, and the $^3H/^{14}C$ ratio of the complex remained constant at 6.36 ± 0.10 (n = 19).

Interaction of dUMP and NPQ with TS from CCRF-CEM Leukemia Cells. A solution containing 2.5 nM enzyme, 50 μM NPQ, and 0.30 μM [6-^{3}H]dUMP (18 Ci/mmol) was incubated at 25 **C** in the standard buffer. At intervals, aliquots were removed, and complex formation was monitored by the nitrocellulose binding assay. Under these conditions, the rate of formation of the complex was a first-order process with $t_{1/2}$ = 102 min. The rate of dissociation of the complex was determined as follows. The complex was formed by incubating 3.0 nM CCRF-CEM TS, 50 μM NPQ, and 0.8 μM [6-^{3}H]dUMP (18 Ci/mmol). After addition of a 200-fold excess of unlabeled dUMP, protein-bound radioactivity was monitored by nitrocellulose binding over a 24-h period. The rate of dissociation was first order with $t_{1/2}$ = 3.2 h.

Formation and Dissociation of FdUMP–NPQ–TS Complexes. Complexes were formed by incubation of 0.08 μM [6-^{3}H]FdUMP, 50 μM NPQ, and 25 nM TS for 18 h at 25 °C. A 200-fold excess of unlabeled FdUMP was added, and at intervals aliquots were assayed for bound radioactivity by the nitrocellulose filter assay. The rate of dissociation was biphasic with 50% of the radioactivity lost with $t_{1/2}$ = 3.0 min and the remainder with $t_{1/2}$ = 100 h.

Discussion

As previously described for TS from other sources,[8,9] NPQ is a potent inhibitor of *L. casei* TS (K_i = 10 nM) which is competitive with respect to CH_2-H_4folate. However, we observed that upon preincubation of NPQ with dUMP and TS, the inhibition becomes noncompetitive with respect to CH_2-H_4folate. Further, incubation of the enzyme with NPQ and [6-^{3}H]dUMP resulted in the slow formation of ternary complexes that could be isolated on nitrocellulose filters or by gel filtration. A similar slow formation of isolable complexes has been observed with dUMP, TS, and 10-ethyl-5,8-dideazafolate.[14] Taken together, the data indicate that after formation of a rapidly reversible dUMP–NPQ–TS complex, there is a slow, unimolecular isomerization to a more stable complex. Unusual features of this complex are the slow rate at which it is formed (k_1 = 0.78 h^{-1}) and the slow rate at which it dissociates (k_1 = 0.026 h^{-1}). It is of interest that dissociation of dUMP from the isolable complex is some fourfold slower than that of FdUMP from the covalent FdUMP–CH_2-H_4folate–TS complex at 25 °C.[15] However, because the formation of the isolable dUMP–NPQ–TS complex is

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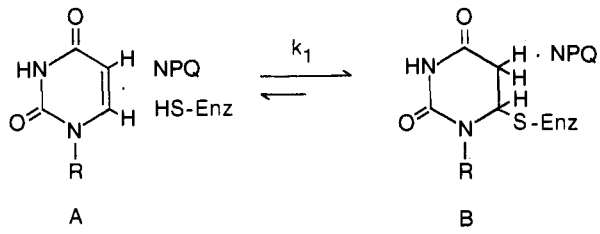

Figure 5. Proposed interaction of dUMP and NPQ indicating (A) the rapidly reversible, noncovalent dUMP–NPQ complex and (B) the isolable complex in which the nucleophilic thiol of the enzyme is covalently bound to dUMP; R = 5-phospho-2-deoxyribosyl.

so slow, the complex is only about 30-fold more stable than the rapidly reversible complex. We have shown that an isolable dUMP–NPQ–enzyme complex is also slowly formed with TS from human CCRF-CEM cells. Dissociation of dUMP from this complex, although still slow with k_{-1} = 0.22 h^{-1}, was about ninefold faster than from the ternary complex formed with the *L. casei* enzyme. The amount of mammalian TS available was limited and all studies described below used the enzyme from *L. casei*.

The formation of the isolable dUMP–NPQ–TS complex was sufficiently slow to permit studies of the rapidly reversible ternary complex by UV difference spectroscopy. The difference spectra of initially formed dUMP–NPQ–TS complexes (vs. NPQ and TS) show maxima at 265 and 325 nm and a minimum at 295 nm. The high wavelength maximum is observed in a number of related ternary complexes of TS, including those containing other 5,8-dideazafolate analogues[14,17] and has been attributed to perturbations of the enzyme and/or ligand chromophores that occur upon binding to the protein.[2,14] Our results show that this perturbation occurs within the rapidly reversible dUMP–NPQ–TS complex and before the slow formation of the isolable complex. The maximum at 265 nm closely corresponds to that of dUMP and the absorbance is equivalent to the amount of nucleotide; thus, the chromophore of dUMP is not modified in the initially formed ternary complex. Further, by titrating the absorbance at 325 nm vs. the dUMP and NPQ concentrations, we have shown that 2 mol of each ligand are bound per mol of dimeric enzyme in both the rapidly reversible and isolable complexes.

Current data suggest that the formation of the isolable ternary complex involves nucleophilic attack of the catalytic thiol group of TS to the 6-position of dUMP, forming the complex shown in Figure 5. The experimental evidence upon which this structural assignment is based is as follows: First, there is a first-order decrease in the absorbance at 265 nm in the difference spectrum of the rapidly reversible dUMP–NPQ–TS complex that proceeds at the *same* rate as the formation of the isolable complex. The loss of absorbance is in accord with the proposed addition across the 5,6-double bond of the uracil moiety. Second, with use of [2-^{14}C,6-^{3}H]dUMP there is an inverse α-secondary kinetic isotope effect of k_H/k_T = 0.83 upon formation of the complex. This demonstrates sp^2 to sp^3 rehybridization of the 6-carbon and is close to the maximal expected for addition of a thiol group to the 6-position of the uracil heterocycle.[15]

Treatment of the complex with $NaDodSO_4$ results in the dissociation of both ligands in an unmodified form. This is not inconsistent with the proposed covalent bond between the enzyme and the 6-position of dUMP since, upon denaturation of the protein, the thiol adduct should undergo a rapid β-elimination to provide dUMP. Indeed, although the 6-position of the uracil heterocycle is susceptible to nucleophilic attack by thiols, most adducts formed are transient and unstable and can only be detected by kinetic methods.[18-20] The finding that NPQ is obtained unchanged upon denaturation of the complex is also consistent with the proposed mechanism (Figure 5). From a chemical standpoint, it is difficult to envision how NPQ could covalently interact with the enzyme and, more so, how it could bo so in reversible fashion. The recovery of unchanged NPQ indicates that it was not covalently attached to the enzyme.

There is much precedence for the formation of a covalent bond between the catalytic nucleophile of TS and the 6-position of pyrimidine nucleotides. It has been well established that such a covalent bond is transiently formed in the normal enzymic reaction and in enzyme-catalyzed conversions of alternate substrates.[2,3] Further, the formation of stable covalent bonds between the enzyme and the 6-position of certain analogues of dUMP is responsible for the potent inhibition of the enzyme by compounds such as FdUMP, NO_2dUMP, and other mechanism based inhibitors of this enzyme.[2,4] Although a transient covalent adduct between dUMP and TS analogous to that depicted in Figure 5 has been detected by isotope exchange,[21] stable binary TS–dUMP adducts have not yet been described. In some manner, the presence of NPQ appears to induce the formation and/or accumulation of a stable covalent complex between TS and the 6-position of dUMP. Two general mechanisms can be proposed for this interaction that are in accord with the α-secondary kinetic isotope effect. First, there may be a rapid preequilibrium formation of low levels of the covalent complex, which then undergoes a slow conversion to provide the isolable complex. Second, the covalent bond could be directly formed from the rapidly reversible complex in a rate-determining step that may include conformational changes of the protein. Although these cannot be distinguished at this time, the finding that a large inverse α-hydrogen isotope effect is observed in the formation of the complex but not in its dissociation would require that the transition state in the latter mechanism be very late. Whatever the exact mechanism is, it is clear that the propargyl group of NPQ is responsible for many of its interesting properties. When this group is omitted, as in 5,8-dideazafolate, the rate of formation of isolable complexes is some 38-fold faster than that of NPQ and the rate of dissociation is increased some 70-fold.

We have also shown that isolable ternary complexes are formed when TS is incubated with FdUMP and NPQ. Although not examined in detail, dissociation of [6-^{3}H]-FdUMP from these complexes has been found to be biphasic, which, as previously described for N^4-hydroxy-dCMP,[22] suggests an asymmetry of the bound subunits. Moreover, the dissociation was exceedingly slow: one-half of the initially bound ligand dissociated with $t_{1/2}$ = 3 min, and the remainder dissociated with $t_{1/2} \simeq$ 100 h. The latter represents the slowest dissociation of any reversible

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complex involving TS thus far reported. Although the structure of the FdUMP–NPQ–TS complex is still under investigation, it is reasonable to believe that reversible covalent bonds between the enzyme and FdUMP are involved. The adduct would be similar to that suggested for the binary FdUMP–TS complex[23] except it would be stabilized by NPQ.

It is of interest to consider how the findings described here might have relevance to the use of NPQ as an anticancer agent. It has been shown that inhibition of TS eventually results in the buildup of intracellular levels of dUMP,[9] which if high enough can prevent inhibition of the enzyme by nucleotide inhibitors such as FdUMP.[24] Furthermore, optimal formation of a tight-binding complex between FdUMP and TS requires a reasonably high level of the reduced folate cofactor, 5,10-CH_2-H_4folate.[25,26] In cells where this cofactor is at low levels, inhibition of TS has been shown to be less than maximal.[26] On the other hand, such conditions would be expected to optimize the binding of a folate analogue such as NPQ to TS because, as shown here, inhibition of the enzyme by NPQ requires dUMP and is competitive with CH_2-H_4folate. In the specific case of NPQ, it remains to be determined whether the slow rate of formation of the stable ternary complex might prove to be an obstacle or an advantage to the effectiveness of NPQ as a drug. According to Figure 5, the rate at which TS is inactivated is a product of k_1 and the concentration of the rapidly reversible ternary complex. The concentration of the latter might be increased by high levels of dUMP, thereby raising the concentration of the binary dUMP–TS complex; it would be decreased by endogenous folates that compete for the binary dUMP–TS complex and thereby lower the rate of inactivation of TS even further. Thus, achieving appreciable inhibition of TS within a reasonable period of time with NPQ may require one or more of the following: (1) prolonged exposure of the target tissue to the drug, with potential accompanying toxicity to normal tissues, (2) concurrent preadministration of other agents designed to achieve low levels of reduced folates in the cells of the target tissue, (3) increasing the intracellular concentration of dUMP, perhaps by coadministration of dUrd or an agent such as methotrexate. The observation that the ternary dUMP–NPQ–TS complex has a very slow rate of dissociation indicates that once it is formed, the inhibition of TS would be prolonged. It would also be of interest to examine the effects of FUra and FdUrd in combination with NPQ that could form FdUMP–NPQ–TS complexes with even slower rates of dissociation. It has also been shown that NPQ is enzymatically converted to polyglutamate forms[27] that would decrease the rate of dissociation of ternary TS complexes even further. Thus NPQ may be effective in some slow-growing tumors that are unresponsive toward other inhibitors of TS, and some of the cytotoxicities toward rapidly proliferating cells might be specifically reversed by rescue and dThd.

Experimental Section

Materials. [6-^{3}H]dUrd (18 Ci/mmol), [2-^{14}C]dUMP (55 mCi/mmol), and [6-^{3}H]FdUMP (20 Ci/mmol) were obtained from Moravek Biochemicals, City of Industry, CA. [6-^{3}H]dUMP was prepared and purified as reported.[28] NPQ was a gift from A. H. Calvert, Institute of Cancer Research, Sutton, Surrey, U.K. 5,10-Dideazafolate was provided by J. B. Hynes, Medical University of South Carolina. Nitrocellulose B45A membranes were from Schliecher and Schuell. Other materials were of the highest grade commercially available.

Thymidylate Synthase. The homogeneous enzyme from methotrexate-resistant *L. casei*[29] was prepared as previously described.[28] The concentration of binding sites was determined by spectrophotometric titration with FdUMP in the presence of CH_2-H_4folate[18] and nitrocellulose binding assays using [6-^{3}H]-FdUMP;[30] the preparation used here was calculated to possess 90% of the theoretical FdUMP binding sites. TS from CCRF-CEM cells was the purified preparation previously described.[31]

Enzyme Assays. Initial velocity measurement of dTMP formation were performed spectrophotometrically at 25 °C.[32] Reaction mixtures contained 50 mM TES (pH 7.4), 25 mM $MgCl_2$, 75 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM dUMP, 0.28 mM CH_2-H_4folate, and limiting enzyme. In standard assays the reaction was initiated by the addtiion of enzyme as previously described;[13] for preincubation experiments the reaction was initiated with CH_2-H_4folate.

Nitrocellulose binding assays of the [6-^{3}H]dUMP–NPQ–enzyme complex were performed by a slight modification of reported procedures for the FdUMP complex.[30] Solutions containing specified concentrations of enzyme, nucleotide, and NPQ in a buffer consisting of 50 mM TES (pH 7.4), 25 mM $MgCl_2$, 1.0 mM EDTA, and 75 mM 2-mercaptoethanol were incubated at 25 °C. At various times, duplicate aliquots (20–75 μL) were applied to moist filters and washed with five 1-mL portions of 20 mM potassium phosphate (pH 7.4). The filters were dissolved in 10 mL of ACS (Amersham), and the radioactivity was determined; dpm values were determined by the external standard ratio method. A filtration efficiency of 90% was determined as previously described.[30]

Difference Spectra. Spectra were recorded and stored on a Hewlett Packard 8450 spectrophotometer interfaced with a HP 85 computer and disk drive. The buffer used was 50 mM TES (pH 7.4), 25 mM $MgCl_2$, 1.0 mM EDTA, and 5.0 mM dithiothreitol. Stored spectra were corrected for light scattering[33] and dilutions followed by subtractions of TS and NPQ to give the final difference spectra representing the TS–NPQ–dUMP complex vs. TS and NPQ.

Chromatography. HPLC was performed with a 4.6 × 250 mM LiChrosorb RP-18 column. NPQ eluted with RV = 23 mL using 5 mM $(n\text{-Bu})_4N^+HSO_4^-$ and 5 mM K_2HPO_4 (pH 7.0) containing 35% MeOH; dUMP eluted with RV = 34 mL using 5 mM $(n\text{-Bu})_4N^+HSO_4^-$ and 5 mM K_2HPO_4 (pH 7.0) containing 3% MeOH. Separation of protein–ligand complexes from free ligands by Sephadex G-25 gel filtration was performed at 4 °C with use of a 1.0 × 27 cm column with 75 mM potassium phosphate (pH 7.4) and 75 mM 2-mercaptoethanol for equilibration and elution.

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