Ligand-Induced Conformational Changes of Thymidylate Synthase Detected by Limited Proteolysis†

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ABSTRACT: Limited tryptic proteolysis was used to investigate conformational changes of thymidylate synthase from *Lactobacillus casei* induced by ligand binding. Most of the identified sites of proteolysis were between R72 and R178, a region that includes a large loop containing residues 90−139 that is absent in thymidylate synthase from most other sources. Hydrolysis at both ends of this region was affected by the presence of dUMP. With dUMP, the preference of initial hydrolysis at the N-terminus of this region was switched from R78 to R72, and hydrolysis at R178 was retarded; the latter effect may be primarily a consequence of steric hinderance since R178 is involved in binding the phosphate moiety of dUMP. Orthophosphate had an effect similar to that of dUMP, not only in retarding hydrolysis at the phosphate binding site (R178) but also in retarding hydrolysis at R78 in favor of R72. Alkylation of the catalytically essential sulfhydryl group of thymidylate synthase by iodoacetamide also resulted in R72 being favored over R78 as a site of initial proteolysis. Its effect on hydrolysis at R178 was, as expected, less than that of dUMP or phosphate. These results indicate that dUMP binding induces conformational changes in thymidylate synthase. Phosphate binding and sulfhydryl alkylation also induce conformational changes similar to those resulting from dUMP binding. While the similarity of the proteolytic behavior of thymidylate synthase in the presence of dUMP or phosphate agrees with the report by Finer-Moore et al. [Finer-Moore, J., Fauman, E. B., Foster, P. G., Perry, K. M., Santi, D. V., & Stroud, R. M. (1993) *J. Mol. Biol. 232*, 1101−1116] that the conformations of the dUMP-bound enzyme and the phosphate-bound enzyme are essentially identical, the results presented here suggest that the conformations of the dUMP-bound and phosphate-bound enzyme differ from the conformation of the native enzyme with neither dUMP nor phosphate ligands.

Thymidylate synthase catalyzes the reductive synthesis of thymidylate[1] from 2′-deoxyuridylate and methylenetetrahydrofolate. The enzyme from *Lactobacillus casei* is a dimer of identical subunits (Dunlap et al., 1971; Loeble & Dunlap, 1972; Maley et al., 1979a), but the stoichiometry of substrate binding and inhibition by sulfhydryl reagents [for example, see Tables I and IV in Lewis and Dunlap (1981)] strongly suggests that it exhibits half-sites reactivity (Danenberg & Danenberg, 1979), in which catalytic activity is apparently expressed by only one half of the putative catalytic sites. However, crystallographic data for native thymidylate synthase from *L. casei*, *Escherichia coli*, T4 phage, and human sources have shown that they are all "perfectly symmetrical dimers" (Monfort et al., 1990; Perry et al., 1990), and thus half-sites reactivity must arise from the induction of conformational changes associated with substrate binding. Although kinetic studies revealed no evidence of this type of extreme negative cooperativity (Daron & Aull, 1978), a flip-flop mechanism in which the enzyme in its catalytic cycle alternates between two active sites need not display cooperative kinetics (Lazdunski, 1972).

A considerable body of evidence for conformational changes associated with catalysis has been obtained by comparison of the native enzyme with stable ternary complexes that presumably mimic the transient ternary complex formed during catalysis. Danenberg and Danenberg (1979) have shown that FdUMP and CH_2-H_4folate binding to one catalytic site induced conformational changes that exposed the other catalytic site. Also, the formation of the ternary complex of thymidylate synthase with FdUMP and CH_2-H_4folate produced changes in the hydrodynamic properties of the enzyme, as measured by gel filtration and density gradient centrifugation, that were consistent with large conformational changes resulting in a more compact structure (Lockshin & Danenberg, 1980). The changes in absorption, fluorescence, and circular dichroic spectra that accompany ternary complex formation are consistent with conformational changes, though other interpretations are possible (Danenberg et al., 1974; Santi et al., 1974; Galivan et al., 1975; Donato et al., 1976; Manavalan et al., 1986). The conformational changes that accompany the formation of ternary complexes of *E. coli* thymidylate synthase with either dUMP or FdUMP and the folate analogue 10-propargyl-5,8-dideazafolate or with FdUMP and CH_2-H_4folate have been described in detail by comparison of its crystal structure with that of the phosphate-bound enzyme (Monfort et al., 1990; Finer-Moore et al., 1990; Matthews et al., 1990a,b).

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[1] Abbreviations: CH_2-H_4folate, N^5,N^{10}-methylenetetrahydrofolate; DPCC, diphenylcarbamylchloride; dUMP, 2′-deoxyuridylate; dTMP, thymidylate; FdUMP, 5-fluoro-2′-deoxyuridylate; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid; TLCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone; PDDF or CB3717, 10-propargyl-5,8-dideazafolate.

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The reports by Kamb et al. (1992a,b) of the crystallographic structures of binary complexes of *E. coli* thymidylate synthase with both mono- and tetraglutamyl folate analogues have shown that most of the major conformational changes accompanying formation of the ternary complexes can be induced by the folate cofactor alone, thus implying that conformational changes accompanying dUMP binding are relatively minor. This implication was supported by Finer-Moore et al. (1993), who found no significant difference between the conformations of the dUMP-bound and the phosphate-bound thymidylate synthase from *L. casei*. However, if the unliganded enzyme is a symmetric dimer, then some conformational changes induced by dUMP binding are required to account for the reported half-sites reactivity. Observations consistent with such changes in conformation include those from stopped-flow kinetic studies of the quenching of tryptophan fluorescence by dUMP, dTMP, and FdUMP binding, which were consistent with a two-step mechanism involving the rapid preequilibrium formation of a binary complex followed by a slower isomerization step (Mittelstaedt & Schimerlik, 1986). The reported changes in the circular dichroic spectra (Leary et al., 1975) and the protection from heat denaturation (Galivan et al., 1976) and enzymatic digestion (Galivan et al., 1977) that accompany substrate or other ligand binding are also consistent with ligand-induced conformational changes. Evidence for the induction of conformational changes in the C-terminal region of *L. casei* thymidylate synthase by dUMP has recently been obtained (Carreras et al., 1994). Using a modified enzyme to which a spin-label was attached to the C-terminus, they showed that dUMP increased the relative population of a conformer having restricted mobility at the C-terminus. Tryptic proteolysis was used in the present study as a probe to investigate the alteration of the conformation of thymidylate synthase induced by ligand binding and to identify regions where those conformational changes occurred. A preliminary account of these results has been presented (Mohsen et al., 1991).

MATERIALS AND METHODS

Materials. Benzene, ethanol, heptane, hexafluoroacetone trihydrate, thiourea, and a 25% solution of trimethylamine were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). N-Tris(hydroxymethyl)methylglycine (Tricine) and pronase A were from Calbiochem-Behring Corp. (La Jolla, CA). Acetonitrile (Optima grade), ethyl acetate (HPLC grade), methanol (HPLC grade), and sodium acetate (HPLC grade) were from Fisher Scientific (Pittsburgh, PA). Phenylisothiocyanate (sequanal grade) was from Pierce Chemical Co. (Rockford, IL). Amethopterin, aprotinin, bovine serum albumin, carbonic anhydrase, chicken ovalbumin, TLCK-treated chymotrypsin, disodium thymidine 5′-monophosphate, disodium 2′-deoxy-5′-fluorouridine 5′-monophosphate, disodium 2′-deoxyuridine 5′-monophosphate, glucagon, guanidine hydrochloride, kallikrein, α-lactalbumin, PTH-amino acid standards, rennin, somatostatin, *Staphylococcus aureus* V8 protease, subtilisin A from *Bacillus licheniformis* (Carlsberg), trifluoroacetic acid, DPCC-treated trypsin (bovine pancreas), and urokinase were from Sigma Chemical Co. (St. Louis, MO). All other chemicals and reagents obtained commercially were reagent grade or better. (±)-Tetrahydrofolic acid was prepared by catalytic hydrogenation of folic acid (Hatefi et al., 1960), was lyophilized and packaged under argon in sealed serum bottles (Caldwell et al., 1973), and was stored at −50 °C until use. CH_2-H_4folate solutions were prepared as described by Lyon et al. (1976). Ultrapure water was obtained with the Milli-Q UF Plus system (Millipore, Bedford, MA).

Enzyme Preparation and Derivatization. Thymidylate synthase was purified from methotrexate-resistant *L. casei* cells (Lyon et al., 1976). Enzyme concentration was determined spectrophotometrically using extinction coefficients at 278 nm of 105 000 M^{-1} cm^{-1} or 1.55 mL mg^{-1} cm^{-1} (Lyon et al., 1976). It was activated immediately before use by dialysis against 0.1 M potassium phosphate buffer, pH 6.8, containing 25 mM 2-mercaptoethanol for at least 12 h at 4 °C. The ternary complex of thymidylate synthase, FdUMP, and CH_2-H_4folate was prepared by incubating 1 mL of a solution containing 0.055 mM activated thymidylate synthase, 0.27 mM FdUMP, and 0.38 mM CH_2-H_4folate at ambient temperature for 10 min. Thymidylate synthase inactivated by treatment with iodoacetamide was prepared by removing 2-mercaptoethanol from solutions of activated enzyme using PD-10 prepacked columns of Sephadex G-25M (Pharmacia Fine Chemicals, Piscataway, NJ). Columns were equilibrated and eluted with 0.1 M potassium phosphate buffer, pH 6.8, purged with argon. Dethiolated enzyme preparations were treated with 1000-fold molar excess of iodoacetamide, and the mixture was incubated in the dark at ambient temperature. The enzyme lost 100% of its activity within 1 h.

Enzyme Assay. Thymidylate synthase activity was measured spectrophotometrically at 30 °C as previously described (Daron & Aull, 1978).

Limited Proteolysis. Activated thymidylate synthase and its ternary complex were prepared for proteolytic digestion by dialyzing solutions (1 mg/mL) against 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM 2-mercaptoethanol (proteolysis buffer) for over 8 h at 4 °C. Iodoacetamide-inactivated thymidylate synthase (1 mg/mL) and its untreated control were dialyzed against argon-purged 50 mM Tris-HCl buffer, pH 7.5, for 12 h in a sealed container under argon at 4 °C. Ligands, when present, were added to digestion mixtures to give final concentrations of 10 mM prior to the addition of protease. Proteolysis was initiated by adding proteases in enzyme/protease ratios from 10:1 to 200:1 (w/w) and was conducted at 30 °C. Samples were withdrawn periodically and either (i) assayed for enzymatic activity, (ii) boiled for 2 min in 50 mM Tris-HCl buffer, pH 6.8, containing 2% sodium dodecyl sulfate and 5% 2-mercaptoethanol for polyacrylamide gel electrophoresis, or (iii) treated by adding TFA to a final concentration of 0.2% and stored at 4 °C for HPLC analysis.

Reverse-Phase HPLC. Peptides from tryptic digestion mixtures were analyzed and purified by reverse-phase chromatography on a Phenomenex Bondclone C18 column (300 × 3.9 mm) with a μBondapak C18 cartridge guard column (Waters) using a Waters 600 Multisolvent Delivery System and a Lambda-Max 481LC spectrophotometer. The column was equilibrated with 95% solvent A (water with 0.1% TFA) and 5% solvent B (80% acetonitrile with 0.08% TFA), and peptides were eluted by a linear gradient from 5% to 58% solvent B over 53 min followed by a 5 min isocratic period and another linear gradient from 58% to 78% solvent B over 20 min at a flow rate of 1.8 mL/min. The column effluent was monitored at 220 nm. Data were

collected and chromatographic peaks integrated using the Maxima 820 computer program (Dynamic Solutions, Ventura, CA). Samples of 100 μL were injected for analysis, and peptides were isolated for identification from repeated 0.5 mL injections containing 2.5 mg of trypsin-digested thymidylate synthase. Individual peptides were collected manually and concentrated with a SpeedVac SC100 centrifuge. Peptide preparations containing >5% contaminants were rechromatographed using the same procedure described above.

Peptide Sequencing. The N-terminal amino acid sequences of purified peptides were determined using the manual Edman degradation method essentially as described by Tarr (1986). PTH-amino acids were identified by HPLC based on the method of Zimmerman and Pisano (1977). An Alltech Econosphere C18 5μ column (250 mm × 4.6 mm) with a μBondapak C18 cartridge guard column was equilibrated with 80% solvent A (0.01 M sodium acetate, pH 4.5) and 20% solvent B (acetonitrile) at a flow rate of 1 mL/min and 62 °C. After the samples were injected, solvent B was increased to 44% in 6 min, kept at 44% for 8 min, increased again to 55% in 1 min, and kept at 55% for 5 min. The eluted PTH-amino acids were detected at 254 nm and identified by comparison of their retention times with those of standard PTH-amino acids.

Determination of Peptide Molecular Masses. Molecular masses of purified peptides were estimated by SDS−PAGE, size exclusion HPLC, and/or mass spectrometry. SDS−PAGE was conducted at ambient temperature according to the method of Schägger and Jagow (1987). Peptide molecular masses were estimated from their migration distances using thymidylate synthase, carbonic anhydrase, α-lactalbumin, aprotinin, and glucagon as molecular mass standards. Size exclusion HPLC was performed on purified peptides using a Phenomenex TSK G2000SW column (300 mm × 7.5 mm) with a guard column of the same material (100 mm × 7.5 mm). The column was equilibrated and eluted with 6 M guanidinium chloride in 0.1 M sodium phosphate buffer, pH 6, at a flow rate of 0.6 mL/min and 25 °C (Kato et al., 1980). Peptides were purified from a 90-min tryptic digestion mixture containing 4 mg of thymidylate synthase; half of each peptide preparation was used for M_r determination by size exclusion HPLC after treatment with iodoacetamide (Kato et al., 1980). Bovine serum albumin, ovalbumin, carbonic anhydrase, α-lactalbumin, aprotinin, glucagon, and somatostatin (50 μg each) were also treated with iodoacetamide and used as molecular mass calibration standards after adjusting mass values for iodoacetamide incorporation. Positive fast atom bombardment mass spectra were obtained using a VG Analytical 70EHF mass spectrometer (Manchester, England). Samples containing purified peptides were dried in a vacuum centrifuge, dissolved in 50% acetic acid, and added to thioglycerol matrix for application to the probe. The scan resolution was 3000 at a 3.5 kV accelerating potential. For each peptide, the mass of the $(M+H)^+$ ion was determined by computer analysis using two cesium iodide reference peaks in the appropriate mass range.

RESULTS

Eight proteases were initially examined as possible probes to detect conformational changes of thymidylate synthase induced by ligand binding. Incubation of thymidylate synthase with either chymotrypsin [100:1 thymidylate synthase/protease (w/w)], kallikrein (20:1), pronase A (100:1), *S. aureus* V8 protease (10:1), subtilisin A (150:1), or trypsin (200:1) over a 2.5 h period resulted in the loss of activity and the concurrent appearance of peptide fragments in SDS−PAGE gels. Thymidylate synthase did not lose activity when incubated with either rennin (10:1) or urokinase (10:1), nor was any evidence of fragmentation observed with SDS−PAGE. The loss of activity during incubation with subtilisin A or trypsin was retarded when dUMP was present in the incubation mixture, but the peptide pattern obtained by SDS−PAGE was not significantly altered by the presence of dUMP. SDS−PAGE patterns of peptides produced by incubating the ternary complex with either chymotrypsin, kallikrein, or trypsin differed from the patterns produced from the native enzyme. Trypsin was selected for further investigations on the basis of these preliminary results and its defined specificity.

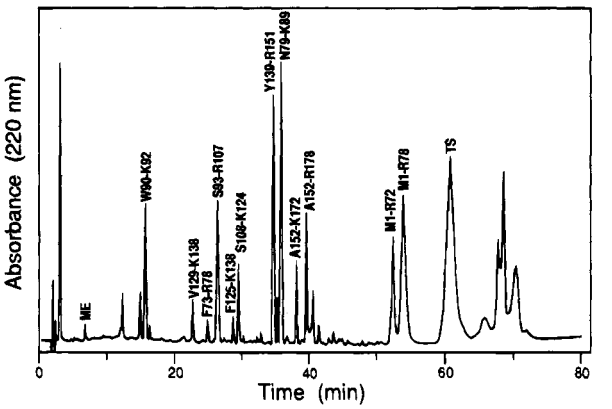

FIGURE 1: Chromatogram of tryptic peptides from native thymidylate synthase. A sample of 100 μg of thymidylate synthase was incubated with 0.5 μg of trypsin for 90 min at 30 °C.

An HPLC chromatogram of the peptides produced by digesting native thymidylate synthase with trypsin for 90 min at 30 °C is shown in Figure 1. Native thymidylate synthase eluted at 60.9 min and 2-mercaptoethanol at 6.7 min. Peptides were isolated and purified by reverse-phase HPLC. They were identified by comparing (i) their N-terminal amino acid sequences, (ii) their molecular masses, (iii) their relative absorbances at 280 nm (which are related to tryptophan and tyrosine content), and (iv) their C-terminal lysine or arginine with the known sequence of *L. casei* thymidylate synthase (Maley et al., 1979b). Peptides that were identified are listed in Table 1. Peptides A152-K157 and E270-K274, which were produced in relatively small quantities, were tentatively identified from their M_r values. Preparations of each of the four major peaks that eluted later than thymidylate synthase were isolated and subjected to SDS−PAGE. Only the preparation from the 70.5 min peak contained a single component with a M_r of 12 700 daltons. Preparations of each of the other late-eluting peaks were mixtures. The 65.9 min peak contained peptides with M_rs of 18 300 and 19 600, the 67.9 min peak contained peptides with M_rs of 4100 and 12 600, and the 68.6 min peak contained peptides with M_rs of 12 100, 22 600, and 30 500. These peptides probably came from the hydrophobic core of thymidylate synthase.

Limited Proteolysis in the Absence of Ligands. The rates of formation of individual peptides were investigated to determine which peptides were produced early in proteolysis.

Table 1: Identification of Tryptic Peptides from *L. casei* Thymidylate Synthase

peptide	R_t (min)	A_{280}	N-terminal sequence	molecular mass SDS-PAGE	molecular mass SE-HPLC	molecular mass MS	molecular mass calculated[a]
M1-R72	52.7	low	MLEQP	7800	7800		8370.4
M1-R78	54.0	low	MLEQP	9400	9000		9164.8
F73-R78	24.9	low	FLLQH			812.4	812.5
N79-K89	36.1	high	NHIWD		1440	1473.8	1473.7
W90-K92	15.7	high	WVK			431.3	431.3
S93-R107	26.4	low	SDEYH			1762.3	1762.7
S108-K124	29.5	low	SQKDP			1978.0	1978.9
F125-K138	28.6	low	FDDRV			1618.6	1618.8
V129-K138	22.7	low	VLHDD			1085.4	1085.6
Y139-R151	34.9	high	YGDLG		1540	1512.6	1512.7
A152-K157[b]	14.9	high				728.3	728.4
A152-K172	38.4	high	AWH[c]			2352.2	2353.2
A152-R178	39.8	high	AWH[c]		3000	3093.1	3094.6
E270-R274[b]	16.3	low				632.2	631.2

[a] Molecular mass calculated according to the monoisotopic values of amino acids (Buko et al., 1983). [b] Tentative identification. [c] Data were inconclusive on the fourth and fifth cycles.

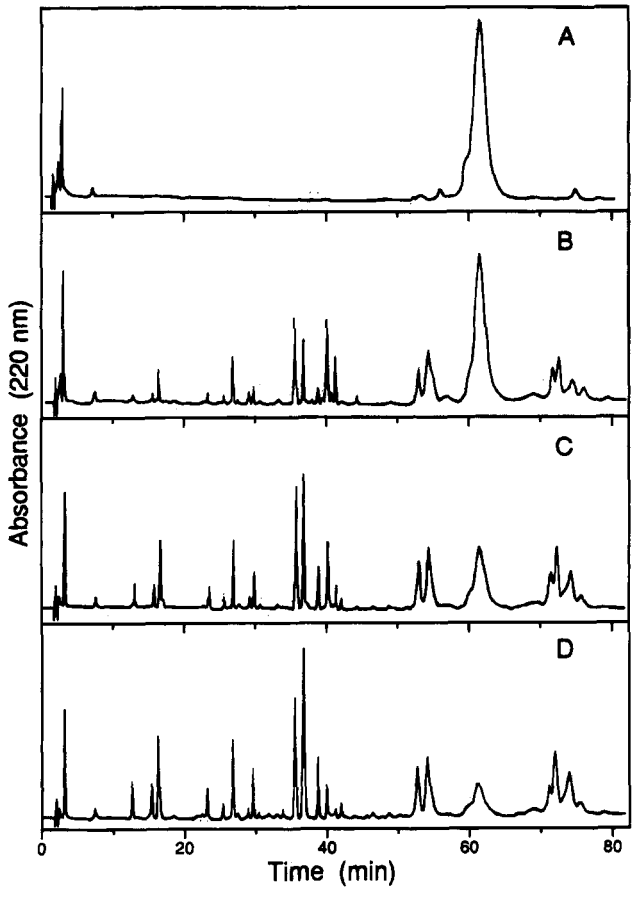

FIGURE 2: Effect of time of digestion on chromatograms of thymidylate synthase peptides. Thymidylate synthase was incubated with trypsin (200:1 w/w) at 30 °C. Samples were withdrawn at 0 min (A), 20 min (B), 80 min (C), and 160 min (D), and proteolysis was stopped by the addition of TFA.

Samples were withdrawn from proteolytic digestion mixtures at 0, 10, 20, 40, 80, 120, 160, and 200 min. Chromatograms of representative samples are shown in Figure 2. Areas of peaks from replicate chromatograms were highly reproducible, and all data shown are averages from two or three chromatograms. In order to compare rates of formation of peptides having different sizes and amino acid content, the relative molar amounts of peptides were estimated from their

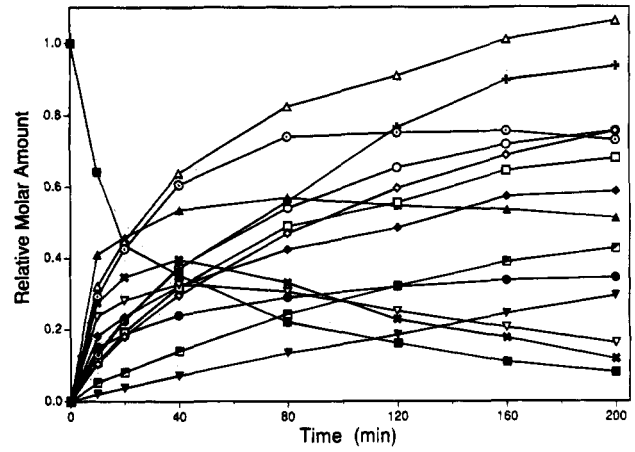

FIGURE 3: Rates of formation of individual tryptic peptides of thymidylate synthase. The relative molar amount of each peptide is plotted as a function of the time of incubation. Relative molar amounts were calculated from molar absorbance values and areas of chromatographic peaks corresponding to each tryptic peptide as described in Results. The relative molar amount of thymidylate synthase at the beginning of the incubation period is assigned a value of 1.0, and amounts of all peptides are expressed relative to this value. Data points at 40 and 80 min are the averages of peak areas from two chromatograms; all other data points are averages of peak areas from three chromatograms. Peptide identification: (●) M1-R72; (▲) M1-R78; (◆) F73-R78; (□) N79-K89; (○) W90-K92; (△) S93-R107; (◇) S108-K124; (▽) F125-K138; (+) V129-K138; (⊙) Y139-R151; (▼) A152-K157; (◩) A152-K172; (×) A152-R178; (■) thymidylate synthase.

peak areas and the molar absorbances of amino acids and their derivatives at 220 nm (Fasman, 1975). The validity of this approach was demonstrated when similar estimates calculated from molar absorbances at 205 nm closely agreed with values obtained from the empirical equation of Scopes (1974). Relative molar amounts of most of the identified peptides are plotted as a function of time of digestion in Figure 3. Over 90% of the thymidylate synthase was hydrolyzed during the 200 min digestion period.

M1-R78 was one of the few peptides observed in chromatograms of the earliest samples, and it was the most abundant peptide after 10 min digestion. Hydrolysis at R78 was probably sufficient to destroy catalytic activity since the initial rapid decrease of both thymidylate synthase activity and the area of its associated peak approximately paralleled the rate of M1-R78 formation. However, the total amount of M1-R78 never exceeded 60% of its hypothetical maximum value due to its further degradation. R72 is the only identified site of hydrolysis in M1-R78, and hydrolysis at this position is probably the first event in the degradation of M1-R78, since approximately equal amounts of M1-R72 and F73-R78 are produced during the early part of the digestion period. The amount of F73-R78, which is not subject to further tryptic hydrolysis, becomes proportionately greater than M1-R72, presumably because of degradation of the latter to peptides that have not been identified. Hydrolysis at R78 was complete between 80 and 120 min of the incubation period as judged by the total amount of M1-R78 and F73-R78 formed. Some M1-R72 may have been produced directly from thymidylate synthase since chromatograms of early samples always showed a minor peak corresponding to M1-R72 along with the M1-R78 peak. Hydrolysis at R78 apparently did not result in unfolding to make K89 readily accessible to trypsin since N79-K89 was produced much more slowly than M1-R78. S93-R107 was also formed

rapidly during the initial phase of the digestion period. Since hydrolysis at R78 and K92 were rapid, peptide N79-K92 was probably formed. The subsequent slower hydrolysis at K89 would account for the approximately equal rates of formation of N79-K89 and W90-K92.

Comparison of peptides S93-R107, S108-K124, and F125-K138 shows that S108-K124 was formed more slowly than either of the others. This result suggests that an undetected, hypothetical peptide, S93-K138, may have been a precursor. Although K110 has not been identified as a site of tryptic hydrolysis from the peptides that have been identified, hydrolysis at this site also may have contributed to the slow rate at which S108-K124 accumulated. The amount of F125-K138 increased rapidly during the initial phase of the digestion but subsequently decreased due to hydrolysis at R128 forming V129-K138.

Y139-R151 and A152-R178 were also among the earlier appearing peptides. Since their amounts were essentially the same in the 10 min sample, they may have been formed from an undetected, hypothetical precursor, Y139-R178, by hydrolysis at R151. The decrease in the amount of A152-R178 after 40 min is the result of its degradation to A152-K157 and A152-K172.

Effect of Nucleotide Ligands on Limited Proteolysis. Chromatograms of thymidylate synthase tryptic hydrolysates were similar when 10 mM dUMP, dTMP, or FdUMP were present in the proteolytic digestion mixture but differed from chromatograms of digestion mixtures lacking nucleotides (Figure 4). The most pronounced effect of nucleotides was the complete suppression of the peak at 54 min corresponding to M1-R78 and the relative enhancement of M1-R72 (52.7 min). Chromatographic peaks corresponding to A152-K172 and A152−R178, which eluted between 38 and 41 min, were also severely suppressed by the presence of nucleotides, while those corresponding to most of the peptides that eluted between 15 and 37 min were essentially unaffected. Nucleotides also suppressed peaks eluting at 12.3 (unidentified) and 14.9 min (tentatively identified as A152-K157) and altered the region of the chromatogram from 63 to 72 min. The presence of dUMP, FdUMP, and to a lesser extent dTMP retarded proteolysis of thymidylate synthase as judged by the relative size of the peak corresponding to thymidylate synthase.

The effect of dUMP on the rates of appearance of individual thymidylate synthase tryptic peptides was investigated more extensively by withdrawing samples at 0, 10, 20, 40, 80, 120, 160, and 200 min from proteolytic digestion mixtures containing 10 mM dUMP. This concentration of dUMP is well above the K_m of 0.7 μM (Daron & Aull, 1978) to ensure that the enzyme was completely saturated during digestion. The protection afforded by dUMP on the rates of loss of enzymatic activity and hydrolysis of thymidylate synthase by trypsin is shown in Figure 5, panels A and B, respectively. The presence of dUMP had a pronounced effect on the rates of production of several peptides. The most striking effect was with M1-R78, whose formation was completely suppressed by dUMP (Figure 5C). Since this effect was accompanied by the concomitant stimulation of M1-R72 and F73-R78 formation (Figure 5D,E), hydrolysis at R72 must have either slightly preceded or occurred almost simultaneously with hydrolysis at R78 when dUMP was present. A second peptide whose rate of formation was greatly reduced by dUMP was A152-R178 (Figure 5F).

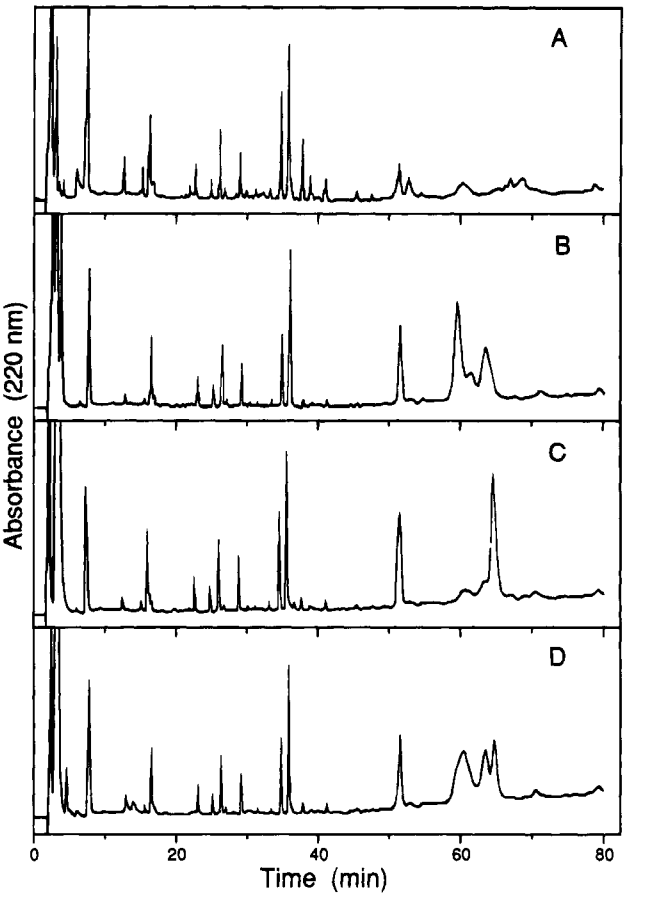

FIGURE 4: Effect of nucleotides on chromatograms of thymidylate synthase peptides. Thymidylate synthase was incubated with trypsin (200:1 w/w) at 30 °C for 270 min in the absence of nucleotides (A) or in the presence of 10 mM dUMP (B), dTMP (C), or FdUMP (D).

dUMP probably retarded hydrolysis at R178, since this residue, as well as R179, is directly involved in binding the phosphate moiety of dUMP (Montfort et al., 1990). The small amount of A152-R178 formed in the presence of dUMP remained essentially unchanged throughout most of the digestion period, probably because the rates of its degradation to A152-K157 and A152-K172 balanced its slow rate of formation. The greatly decreased rates of A152-K157 and A152-K172 formation in the presence of dUMP (Figure 5G,H) are consistent with this interpretation, and they also indicate that hydrolysis at R151 and R178 preceded hydrolysis at K157 and K172. Two other peptides whose rates of formation were decreased by dUMP were F125-K138 and the adjacent Y139-R151 (Figure 5I,J). Their coincident suppression suggests that the presence of dUMP decreased the rate of hydrolysis at K138. Also consistent with this interpretation is the observation that S108-K124 was produced faster than F125-K138 in the presence of dUMP (whereas F125-K138 was produced faster than S108-K124 when dUMP was absent, as previously mentioned), indicating that hydrolysis at K124 must have preceded hydrolysis at K138. The decline in the amount of F125-K138 observed after 20 min probably resulted from hydrolysis at R128 to produce V129-K138 (Figure 5K), which exhibited a slight lag period in its rate of formation. However, the rates of formation of both F125-K138 and V129-K138 were approximately the same, and the absence of a comparable effect of dUMP on V129-K138 production suggests that an

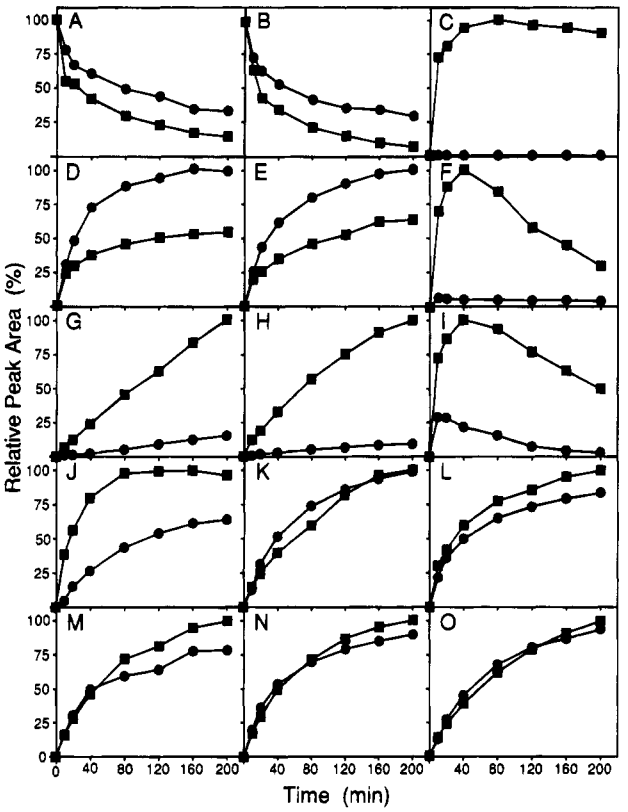

FIGURE 5: Effect of dUMP on rates of formation of peptides from thymidylate synthase. Panel A shows the loss of thymidylate synthase activity; all other panels show the relative areas of chromatographic peaks plotted as a function of the time of incubation with trypsin. The largest area is set equal to 100%. Panels B−O are thymidylate synthase (B), M1-R78 (C), M1-R72 (D), F73-R78 (E), A152-R178 (F), A152-K157 (G), A152-K172 (H), F125-K138 (I), Y139-R151 (J), V129-K138 (K), S93-R107 (L), N79-K89 (M), W90-K92 (N), and S108-K124 (O). Data are from digestion mixtures that either lacked (■) or contained (●) 10 mM dUMP. Areas corresponding to 100% are 21692, 2391, 2528, 262, 884, 439, 732, 151, 1537, 318, 925, 1970, 984, and 573 μV·s for panels B−O, respectively.

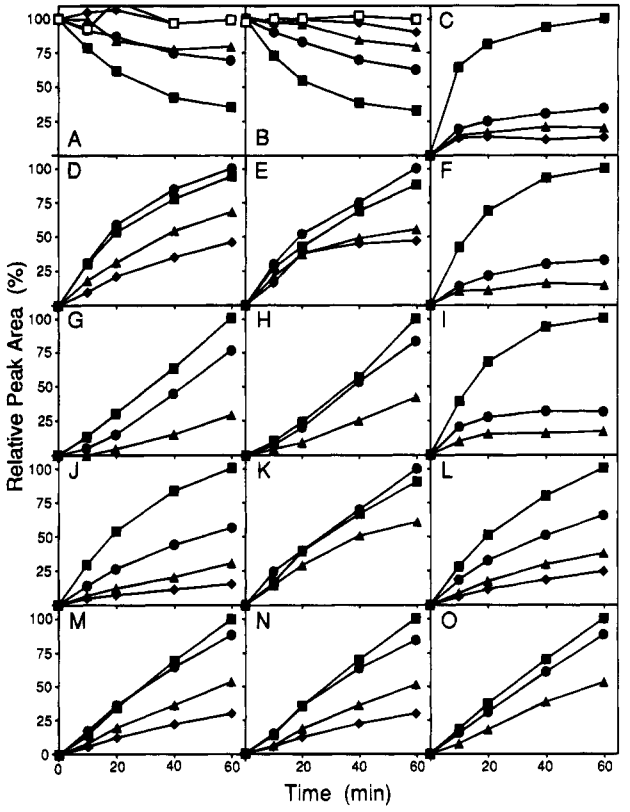

FIGURE 6: Effect of phosphate on rates of formation of peptides from thymidylate synthase. Panel A shows the loss of thymidylate synthase activity; all other panels show the relative areas of chromatographic peaks plotted as a function of the time of incubation with trypsin. The largest area is set equal to 100%, except for thymidylate synthase where 100% equals its activity (A) and the area of its chromatographic peak (B) at zero time in digestion mixtures that lacked phosphate. Panels B−O are thymidylate synthase (B), M1-R78 (C), M1-R72 (D), F73-R78 (E), A152-R178 (F), A152-K157 (G), A152-K172 (H), F125−K138 (I), Y139-R151 (J), V129-K138 (K), S93-R107 (L), N79-K89 (M), W90-K92 (N), and S108-K124 (O). Data are from digestion mixtures that either lacked (■) or contained 10 mM (●), 20 mM (▲), 40 mM (◆), or 80 mM (□) potassium phosphate. Areas corresponding to 100% are 22603, 28857, 7469, 948, 11013, 711, 2296, 1823, 11431, 1588, 6022, 9849, 4462, and 2205 μV·s for panels B−O, respectively. The value of the point that lies outside panel A is 113% (20 min, 80 mM phosphate).

alternate route not involving F125-K138 as a precursor may have also contributed to its formation. Peptides whose rates of formation were essentially unaffected by dUMP were S93-R107, N79-K89, W90-K92, and S108-K124 (Figure 5L−O), though dUMP may have slightly suppressed S93-R107 (Figure 5L).

Effect of Phosphate on Limited Proteolysis. The effect of phosphate on the rate of appearance of the thymidylate synthase tryptic peptides was investigated because earlier studies had shown that phosphate influenced the rate at which thymidylate synthase activity is lost due to tryptic digestion (Aull et al., 1974; Galivan et al., 1977), and because phosphate has been shown to decrease nucleotide affinity (Galivan et al., 1976) and perhaps even the number of FdUMP binding sites. While FdUMP has one binding site in phosphate buffer, in Tris buffer two sites were identified by equilibrium dialysis (Galivan et al., 1976) but only one by calorimetry (Beaudette et al., 1980). Phosphate concentration had a pronounced effect on the rate of proteolysis (Figure 6) and loss of activity. Generally, the rates of formation of all identified peptides were suppressed with potassium phosphate concentrations of 20 mM and greater. Figure 6B shows there was essentially no decrease in the area of the thymidylate synthase peak in 80 mM phosphate, and peptide production was so severely suppressed that chromatographic peaks were barely detectable even after 60 min digestion. Therefore, data for individual peptides formed in 80 mM phosphate are not shown. With the exception of peptides eluting later than thymidylate synthase, the effect of 10 mM phosphate on individual peptide production was qualitatively similar to that with dUMP. M1-R78 was clearly detectable though its rate of formation was greatly reduced (Figure 6C); however, the rates of formation of M1-R72 and F73-R78, which were greatly enhanced in the presence of dUMP, were only slightly increased in 10 mM phosphate (Figure 6D,E). As expected, A152-R178 production was severely suppressed by phosphate (Figure 6F), since R178 is a binding site for phosphate as previously noted. In contrast, A152-K172 production was nearly unaffected by 10 mM phosphate (Figure 6H) but was severely suppressed by dUMP. Rates of formation of F125-K138 and Y139-R151, which were severely suppressed by dUMP, were also suppressed in 10 mM phosphate (Figure 6I,J). The effects of 10 mM phosphate on the rates of formation of all other

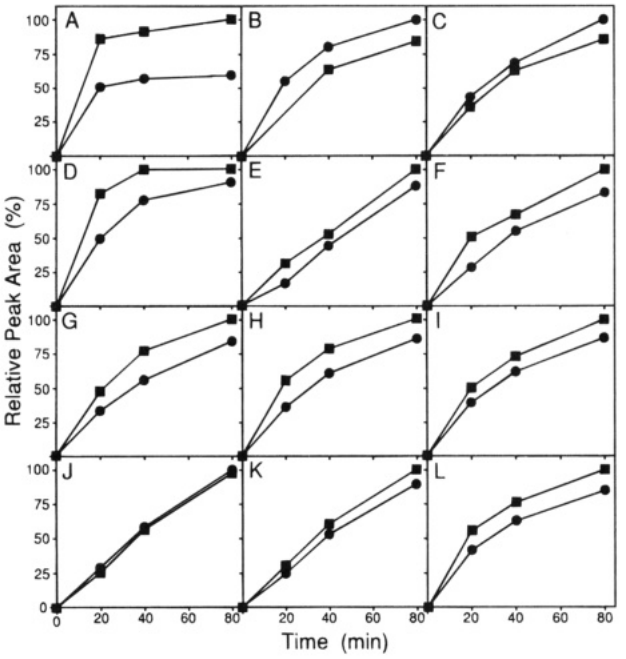

FIGURE 7: Effect of treatment of thymidylate synthase with iodoacetamide on rates of formation of peptides. Relative areas of chromatographic peaks are plotted as a function of the time of incubation with trypsin. The largest area is set equal to 100%. Panels are M1-R78 (A), M1-R72 (B), F73-R78 (C), A152-R178 (D), A152-K157 (E), A152-K172 (F), F125-K138 (G), Y139-R151 (H), S93-R107 (I), N79-K89 (J), W90-K92 (K), and S108-K124 (L). Data are from digestion mixtures with either native thymidylate synthase (■) or iodoacetamide-treated thymidylate synthase (●). Areas corresponding to 100% are 3047.2, 498.5, 98, 621.8, 76.2, 265.3, 89.9, 944.6, 626.8, 776.4, 444.3, and 293.7 μV·s for panels A−L, respectively.

peptides shown in Figure 6 were very similar to those found with dUMP.

Effect of Sulfhydryl Alkylation on Limited Proteolysis. Although there is some disagreement about the precise number of iodoacetamide molecules that react with thymidylate synthase, the number seems to be in the range of 1 (Leary et al., 1975) to 1.5 or 1.6 (Plese & Dunlap, 1977) even after incubation for periods much longer than needed for complete inactivation. It is not clear why iodoacetamide does not react with each of the catalytically essential sulfhydryl groups in the two identical subunits, unless reaction at one catalytic site induces conformational changes that block or limit reaction with the sulfhydryl group on the other subunit. Such a conformational change might be similar to that induced by dUMP. To examine this possibility, we subjected iodoacetamide-treated thymidylate synthase to trypsin proteolysis. Like the results obtained in the presence of dUMP, the rate of formation of M1-R78 was decreased (Figure 7A) and the rates of formation of M1-R72 and F73-R78 were slightly increased (Figure 7B,C) by alkylation of the sulfhydryl groups, although the degree was not as great as that observed with dUMP. Rates of formation of A152-R178, F125-K138, and Y139-R151 were also lower than with untreated native enzyme (Figure 7D,G,H), but the effect was much less than that produced by dUMP. Like dUMP, alkylation had little or no effect on the rates of production of N79-K89 and W90-K92 (Figure 7J,K).

Effect of Folates on Limited Proteolysis. The addition of 10 mM methotrexate to tryptic digestion mixtures almost completely suppressed hydrolysis. Only slight evidence of

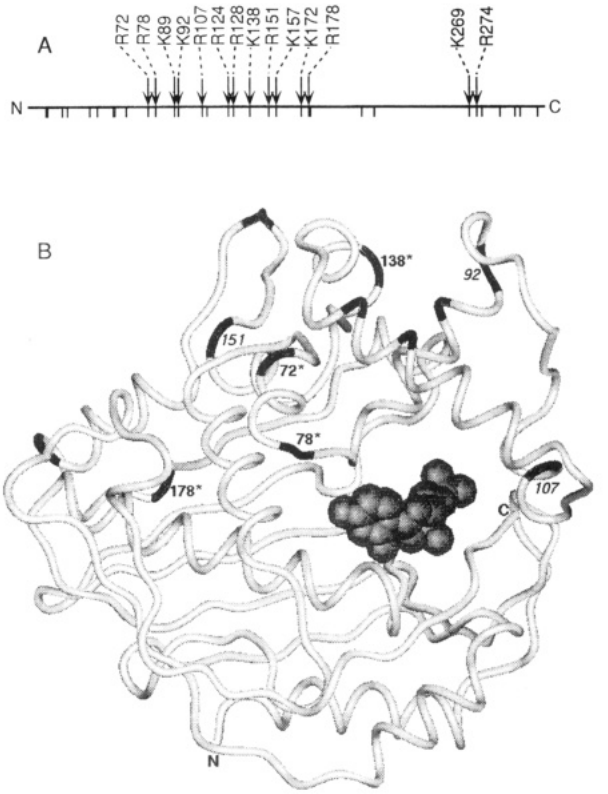

FIGURE 8: Identified sites of tryptic hydrolysis in thymidylate synthase. (A) The horizontal line represents the linear sequence of amino acids in thymidylate synthase from *L. casei*. Vertical marks below the line show the positions of all lysine and arginine residues. Arrows above the line show those positions that have been identified as sites of tryptic hydrolysis. (B) The conformation of the binary complex of dUMP with thymidylate synthase is from coordinates obtained from the Brookhaven Protein Data Bank reference code 1THY (Finer-Moore et al., 1993). The diagram was obtained using Quanta software (Polygen/Molecular Simulations, Waltham, MA) with a Silicon Graphics Indigo 2 workstation. Only one enzyme subunit is shown, and dUMP is shown as a CPK model. Arginine residues that bind the phosphate of the dUMP shown, i.e., R178 and R179, are on the subunit that is not shown. Sites of tryptic hydrolysis from R72 to R178 are shown by black bands in the ribbon diagram. Sites that are potentially initial sites of hydrolysis are identified by residue number. Bold residue numbers with asterisks identify sites whose rates of hydrolysis were altered by the presence of dUMP.

hydrolysis could be seen after 80 min incubation with 200:1 enzyme/trypsin (data not shown). The ternary complex of FdUMP, CH_2-H_4folate, and thymidylate synthase showed only slight evidence of hydrolysis after 15 h incubation with 10 times the usual amount of trypsin (20:1) (chromatogram not shown). These results are in agreement with those of Kamb et al. (1992b) and earlier reports showing that folates cause major conformational changes in thymidylate synthase.

DISCUSSION

The peptides that have been identified reveal that R72, R78, K89, K92, R107, K124, R128, K138, R151, K157, K172, R178, K269, and R274 are sites of tryptic hydrolysis (Figure 8A). Twelve of these sites are in a region that includes a 50-residue loop which is present only in thymidylate synthase from *L. casei* and *S. aureus* transposon Tn4003; the other two sites, viz., K269 and R274, are within 50 residues of the C-terminus. The 12 sites from R72 to R178 are all of the possible tryptic sites within this region except for K110; peptides produced by hydrolysis at this remaining

site have not been identified. As expected, the identified sites of hydrolysis lie in hydrophilic regions; only R72 has a positive hydropathic index with a seven-residue span (Kite & Doolittle, 1982), and all sites appear to be relatively exposed (Hardy et al., 1987; and Figure 8B). It has been suggested that R78 is involved in electrostatic binding of one of the glutamyl residues of the cofactor analogue polyglutamyl-10-propargyl-5,8-dideazafolate (Kamb et al., 1992a), so this site should be exposed in the absence of a folate. The hydropathic index of K110 is lower than that of any of the residues at sites that were hydrolyzed, and there is no apparent reason why tryptic hydrolysis should not have occurred at that site also. The peptide tentatively identified as E270-R274 is located in helix K (residues 266−273), which is on the protein surface and moves about 1 Å during the conformational changes that accompany formation of the ternary complex (Montfort et al., 1990) or binding with a folate analogue (Kamb et al., 1992b).

M1-R72 contains nine potential sites of tryptic hydrolysis: K11, K12, K20, R23, R37, K42, K50, K51, and K58. Although some hydrolysis at these sites cannot be completely excluded, this peptide must be relatively resistant to hydrolysis since there was very little decrease in its concentration after long periods (200 min) of digestion. Most of the potential sites are in hydrophilic regions; K11, K12, and K58 have positive hydropathic indices (seven-residue span), but their values are no greater than that of R72, which is hydrolyzed. Possible explanations for the apparent resistance of M1-R72 to further tryptic hydrolysis include the maintenance of its secondary structure or its continued contact with the enzyme after hydrolysis at R72, either of which could have prevented or retarded attack by trypsin.

dUMP had a profound effect on the rates of formation of peptides M1-R78 and A152-R178. M1-R78 is formed by hydrolysis at R78, and dUMP seemed to switch the preference of initial tryptic hydrolysis from R78 to R72. These residues, shown in Figure 8B, are the second and last residues of helix C (IRFLLQHR), which contains L74, a residue that is conserved in all reported sequences of thymidylate synthase (Perry et al., 1990). A152-R178 is formed by hydrolysis at R151 and R178. R151 is present in all but one of the reported sequences of thymidylate synthase; it is the penultimate residue in helix G (VYGSQWRA) and the last residue of a highly conserved region that includes helix G and residues adjacent to its N-terminus. R178 and R179 are located just before β sheet v; all reported sequences have arg·arg in this location. Since these residues are involved in binding the phosphate moiety of dUMP occupying the catalytic site of the other subunit, it is not surprising that peptide formation was severely restricted by dUMP. Direct steric blocking of R179 by dUMP is probably the major factor in restricting A152-R178 formation, but possible contributions by conformational changes may also be involved. dUMP had little effect on the rate of formation of N79-K89 or on those of W90-K92, S93-R107, and S108-K124, which comprise most of the large loop in the *L. casei* enzyme (residues 90−139). There are no corresponding residues in enzymes from *E. coli*, *Bacillus subtilis*, and phage ϕ3T; vertebrates, yeasts, protozoa, and viruses lack residues 102−139, and phage T4 lacks residues 98−139. However, F125-K138 is also a part of the loop, but its rate of formation was suppressed by dUMP. K138, which appears to be the residue whose hydrolysis is suppressed by dUMP, is the next to last residue in the loop and is adjacent to a highly conserved region (Figure 8B).

The complete suppression of M1-R78 by dUMP indicates that both subunits of thymidylate synthase have similar conformations in the region including R72 and R78, which agrees with the reported symmetry of the dUMP-bound enzyme observed by Stroud and co-workers (Finer-Moore et al., 1993). However, they reported that dUMP was bound to both active sites, in contrast to previous studies by others indicating that only one dUMP was bound to each enzyme dimer (Galivan et al., 1976; Leary et al., 1975). Although an explanation for that discrepancy is not apparent, our results involving M1-R78 and M1-R72 would be consistent with dUMP binding to both subunits, which would be expected to produce identical changes in the conformation of each subunit. On the other hand, if only one dUMP is bound per dimer as previously reported, then the conformational changes induced by dUMP binding which alter protease accessibility to R78 and R72 must be transmitted to the "unliganded" subunit as well; otherwise, some M1-R78 would have been formed since R78 is readily accessible in the native dimer.

It is not surprising that phosphate repressed the formation of A152-R178 since R178 is at the phosphate binding site, but its effect on rates of formation of M1-R78, M1-R72, and F73-R78 suggests that it also induced conformational changes similar to those induced by dUMP binding. The similarity of the effects of dUMP and phosphate is consistent with the finding of Finer-Moore et al. (1993) that the crystallographic structures of dUMP-bound and phosphate-bound thymidylate synthase were not significantly different. There are two possible explanations for their finding: (i) neither phosphate nor dUMP induced conformational changes or (ii) they both induced essentially the same changes. The results presented here suggest that the latter possibility is correct and that the conformations of the dUMP-bound and phosphate-bound enzyme differ from the conformation of the native enzyme having neither dUMP or phosphate ligands. The suggestion that interaction between thymidylate synthase and dUMP does not induce conformational changes (Kamb et al., 1992b) resulted from considering the phosphate-bound enzyme as "unliganded" (Monfort et al., 1990). It should be noted that recent electron paramagnetic resonance studies showing that dUMP induced conformational changes, i.e., altered the relative population of conformers differing in their mobility in the C-terminal region, were not conducted in phosphate buffer (Carreras et al., 1994). The resistance of thymidylate synthase in the presence of methotrexate and of the ternary complex to tryptic proteolysis is consistent with the report that most of the difference in conformation between the native enzyme and the ternary complex can also be induced by cofactor binding in the absence of dUMP (Kamb et al., 1992b). Finally, the results with iodoacetamide-treated thymidylate synthase suggest that alkylation of cysteine 198 may also result in conformational changes similar to those induced by dUMP and, if conformational changes are transmitted between subunits, may help to explain why less than 2 mol of iodoacetamide are incorporated into the enzyme dimer even after extensive treatment.

The results presented here demonstrate a conformational change induced in both subunits of thymidylate synthase as a consequence of binding dUMP or phosphate or alkylation by iodoacetamide. The conformational change, though

perhaps subtle, was readily detected, particularly in helix C near residues R72 and R78. This finding suggests a focus for future investigations of conformational changes associated with thymidylate synthase function, including changes which may explain the half-sites activity exhibited by the enzyme.

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