Mechanism of Inhibition of Mammalian Tumor and Other Thymidylate Synthases by N^4-Hydroxy-dCMP, N^4-Hydroxy-5-fluoro-dCMP, and Related Analogues[†]

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Received March 30, 1990; Revised Manuscript Received July 20, 1990

ABSTRACT: N^4-Hydroxy-dCMP (N^4-OH-dCMP), N^4-methoxy-dCMP (N^4-OMe-dCMP), and their 5-fluoro congeners (syntheses of which are described) were all slow-binding inhibitors of Ehrlich carcinoma thymidylate synthase (TS), competitive with respect to dUMP, and had differing kinetic constants describing interactions with the two TS binding sites. N^4-OH-dCMP was not a substrate (no dihydrofolate produced; no tritium released with 5-^{3}H-labeled molecule), and its inactivation of TS was methylenetetrahydrofolate-dependent, hence mechanism-based, with arrest of a step posterior to addition of cofactor and blocking abstraction of the C(5) hydrogen. K_i values for N^4-OH-dCMP and its 5-fluoro analogue were in the range 10^{-7}–10^{-8} M, 2–3 orders of magnitude higher for the corresponding N^4-OMe analogues. The 5-methyl analogue of N^4-OH-dCMP was 10^4-fold less potent, pointing to the anti rotamer of the imino form of exocyclic N^4-OH, relative to the ring N(3), as the active species. This is consistent with weaker slow-binding inhibition of the altered enzyme from 5-FdUrd-resistant, relative to parent, L1210 cells by both FdUMP and N^4-OH-dCMP, suggesting interaction of both N^4-OH and C(5)–F groups with the same region of the active center. Kinetic studies with purified enzyme from five sources, viz., Ehrlich carcinoma, L1210 parental, and 5-FdUrd-resistant cells, regenerating rat liver, and the tapeworm *Hymenolepis diminuta*, demonstrated that addition of a 5-fluoro substituent to N^4-OH-dCMP increased its affinity from 2- to 20-fold for the enzyme from different sources. With the Ehrlich and tapeworm enzymes, N^4-OH-FdCMP and FdUMP were almost equally effective inhibitors.

Thymidylate synthase (EC 2.1.1.45) catalyzes the formation of dTMP, by methylation of dUMP with concomitant conversion of N^5,N^{10}-methylenetetrahydrofolate to dihydrofolate (Santi & Danenberg, 1984). Since dUMP analogues that inhibit the enzyme, like 5-FdUMP[1] and 5-(trifluoromethyl)-dUMP, are active chemotherapeutic agents (Danenberg, 1977; Heidelberger et al., 1983), new potent inhibitors and their mechanism(s) of action are of continuing interest.

Among the dUMP analogues that are good inhibitors of thymidylate synthase, most involve some modification at the C(5) position of the pyrimidine ring. One interesting exception is N^4-hydroxy-dCMP, which has been reported to inhibit the enzyme from avian (Lorenson et al., 1967) and bacterial (Goldstein et al., 1984) sources. Inhibition is competitive with respect to dUMP, is time- and CH_2FH_4-dependent (Lorenson et al., 1967; Goldstein et al., 1984), and is accompanied by formation of a ternary complex, N^4-hydroxy-dCMP–CH_2FH_4–enzyme, with apparently differing interactions between the low molecular weight components and each of the two enzyme subunits (Goldstein et al., 1984).

To better appreciate the mechanism of inhibition of thymidylate synthase by N^4-OH-dCMP, we compared the interactions of this analogue and some derivatives, N^4-OH-FdCMP, N^4-OMe-dCMP, N^4-OMe-FdCMP, N^4-OH-5-methyl-dCMP, and N^4-OMe-5-methyl-dCMP, with the enzyme isolated from Ehrlich ascites carcinoma, and parental

Table I: R_f Values of Investigated Compounds on Merck Cellulose F$_{254}$ Plates[a]

compd	R_f in solvent					
	A	B	C	D	E	F[b]
dCyd						0.84
dCMP	0.79	0.12	0.63	0.36	0.47	0.63
dCDP						0.53
dCTP						0.45
5-methyl-dCMP	0.54	0.15	0.65			
N^4-hydroxy-dCMP	0.76	0.23	0.46	0.46	0.54	
				0.40[b]		
N^4-methoxy-dCMP	0.87	0.12	0.35	0.61	0.64	
N^4-hydroxy-5-methyl-dCMP	0.79	0.18	0.33	0.48	0.42	
N^4-methoxy-5-methyl-dCMP	0.66	0.59	0.38			
N^4-hydroxy-5-fluoro-dCMP				0.44[b]		
N^4-methoxy-5-fluoro-dCMP				0.54[b]		

[a] The following solvent systems (v/v) were used: (A) 2-propanol–H_2O–25% NH_4OH (4:4:1); (B) 1 M ammonium acetate–ethanol (2:5); (C) saturated $(NH_4)_2SO_4$–H_2O (4:1); (D) 1% $(NH_4)_2SO_4$-2-propanol (1:2); (E) saturated $(NH_4)_2SO_4$-2-propanol–phosphate, pH 7.4 (79:2:19); (F) methanol–NH_4OH–H_2O (6:1:2). [b] Whatman 3MM paper.

and FdURd-resistant L1210, cells, as well as from regenerating rat liver and the tapeworm *Hymenolepis diminuta*.

MATERIALS AND METHODS

Hydroxylamine and methoxyamine were products of Aldrich (Milwaukee, WI). Snake venom phosphodiesterase was obtained from Sigma (St. Louis, MO). Details of thin-layer and

[1] Abbreviations: N^4-OH-dCyd, N^4-hydroxydeoxycytidine; N^4-OH-dCMP, N^4-hydroxy-dCMP; N^4-OMe-dCMP, N^4-methoxy-dCMP; N^4-OH-FdCMP, N^4-hydroxy-5-fluoro-dCMP; N^4-OMe-FdCMP, N^4-methoxy-5-fluoro-dCMP; CH_2FH_4, N^5, N^{10}-methylenetetrahydrofolate; FdUrd, 5-fluorodeoxyuridine; FdUMP, 5-fluorodeoxyuridylate.

paper chromatography are given in Table I. The pK values for protonation and/or dissociation were all determined by spectrophotometric titration. Solvents, buffers, and other reagents were of analytical grade.

Synthesis of Inhibitors. These were all prepared on a small scale by modifications of known procedures, and identification was based on chromatography, spectral properties at various pH values, and pK_a values.

5-Fluoro-N^4-hydroxy-2'-deoxycytidylate (N^4-OH-FdCMP). To 3 mL of an aqueous solution of the disodium salt of 2'-deoxy-4-thio-5-fluorouridylate (40 mg, 0.12 mmol), synthesized as previously described (Dzik et al., 1987), was added 40 mg (0.59 mmol) of $NH_2OH \cdot HCl$ in 8 mL of CH_3OH, and the mixture was stirred for 60 h at 37 °C. It was then concentrated to a small volume under reduced pressure and deposited on a half-sheet of Whatman 3MM, followed by development with solvent D. The major band ($R_f = 0.44$, Table I) was eluted with water and brought to small volume under reduced pressure; the product was precipitated with methanol–acetone and dried over P_2O_5 under vacuum to give 19 mg (48%): λ_{max} (pH 7) 267 nm; λ_{max} (6 N HCl) 269 nm; A_{max} (6 N HCl)/A_{max} (pH 7) 1.4; ϵ/P 1.01; pK_a ≈0.7 [cf. reported value of 0.7 for the nucleoside (Wempen et al., 1968)].

5-Fluoro-N^4-methoxy-2'-deoxycytidylate (N^4-OMe-FdCMP). This was prepared as above with NH_2OCH_3 in place of NH_2OH, in 57% yield: λ_{max1} (pH 7) 275 nm; λ_{max2} (pH 7) 240 nm; λ_{max} (6 N HCl) 297 nm; A_{max} (6 N HCl)/A_{max1} (pH 7) 1.2; ϵ/P 1.02; pK_a ≈0.8.

[5-^{3}H]dCMP. This is not available commercially and was therefore prepared by phosphodiesterase hydrolysis of the triphosphate. To a solution of 1 mL of [5-^{3}H]dCTP (from Amersham; ≈15 Ci/mmol) in 1 mL of 0.1 M Tris-HCl buffer, pH 8.8, was added 0.005 unit of snake venom phohphodiesterase (from Sigma, St. Louis, MO), and the course of hydrolysis was followed by chromatography on Whatman 3MM. Following incubation for 24 h at 37 °C, the yield of the 5'-monophosphate was 86%. This product was isolated by chromatography on Whatman 3MM with solvent F (Table I), eluted with water, and dried over P_2O_5 under vacuum to yield 0.5 mCi of product.

N^4-Hydroxy[5-^{3}H]dCMP was prepared by a modification of the procedures of Maley and Maley (1964) and Goldstein et al. (1984). A solution of 0.5 mCi of [5-^{3}H]dCMP in 1.9 mL of 1 N NH_2OH in neutral 68% aqueous methanol was stored at 37 °C for 48 h. The solution was brought to dryness under reduced pressure, followed by addition of 1 mL of glacial acetic acid. The resulting suspension was stirred at 37 °C for 40 min and then filtered. The filtrate was brought to dryness, and the residue was dissolved in 0.5 mL of H_2O, deposited on Whatman 3MM, developed with 1% $(NH_4)_2SO_4$–2-propanol (1:2 v/v), and isolated by elution with water in 60% yield.

N^4-Hydroxy-dCMP and N^4-methoxy-dCMP were prepared as for N^4-hydroxy[5-^{3}H]dCMP, with NH_2OH and NH_2OCH_3, respectively, in 45% and 56% yields.

5-Methyl-N^4-hydroxy-dCMP and 5-methyl-N^4-methoxy-dCMP were prepared by treatment of 5-methyl-dCMP with NH_2OH and NH_2OCH_3 as described elsewhere (Janion & Shugar, 1965), in 45% and 40% yields, respectively.

(±)-L-Tetrahydrofolic acid was prepared by catalytic hydrogenation of folic acid (Fluka, Buchs, Switzerland) as described by Lorenson et al. (1967) except that 2-mercaptoethanol (Serva, Heidelberg, FRG) was used in place of 2,3-dimercaptopropanol.

[5-^{3}H]dUMP (≈15 Ci/mmol) from Amersham (Amersham, U.K.) was purified by ion exchange chromatography on

a Dowex 1-formate column (Lorenson et al., 1967). The commercial preparation was diluted with water and deposited on the column, and after a wash with 0.1 N formic acid, [5-^{3}H]dUMP was eluted with 4 N formic acid, the solvent evaporated, and the residue dissolved in 2% aqueous ethanol, with cold dUMP (Sigma, St. Louis, MO) added to the desired specific radioactivity, neutralized, and frozen.

Cell Lines. Mouse Ehrlich ascites carcinoma and leukemia L1210 cells were maintained, harvested, and stored as described by Rode et al. (1984). An FdUrd-resistant L1210 cell line was developed as described by Jastreboff et al. (1983) for Ehrlich ascites carcinoma cells. The resulting line was maintained as for parental L1210 cells, except that the cell-bearing mice were treated with FdUrd (75 mg/kg injected ip as 0.5 mL of PBS solution) on the fourth day following transplantation.

Thymidylate Synthase. Electrophoretically homogeneous preparations of the enzyme from Ehrlich ascites carcinoma cells were obtained as previously reported (Jastreboff et al., 1982). Thymidylate synthases from both parental and FdUrd-resistant L1210 cells were purified to apparent electrophoretic homogeneity by affinity chromatography on immobilized N^{10}-formyl-5,8-dideazafolate, followed by concentration on DEAE-cellulose (Rode et al., 1979). The resulting preparations, with specific activities of 0.26 and 0.35 μmol min^{-1} mg^{-1} and subunit molecular weights (mean ± SEM, number of experiments in parentheses), determined by SDS electrophoresis, of 32900 ± 200 (3) and 30400 ± 200 (3), respectively, are described in more detail elsewhere (Zieliński et al., 1990). Highly purified preparations of thymidylate synthase from regenerating rat liver (sp act. 1.5 μmol min^{-1} mg^{-1}) and the tapeworm *H. diminuta* (sp act. 0.9 μmol min^{-1} mg^{-1}) are also reported elsewhere (Ciesla et al., 1990).

Enzyme Assay. Tritium release from [5-^{3}H]dUMP in the reaction catalyzed by thymidylate synthase was determined as previously described (Rode et al., 1984), all measurements being done in triplicate. N^4-OH-dCMP and its analogues were added to the reaction mixture as neutral aqueous solutions.

Kinetic Studies. To identify the type of inhibition involved, the effect of the various analogues on the dependence of reaction rate on dUMP concentration, in the form of Lineweaver–Burk plots, was analyzed as previously reported (Rode et al., 1984).

Quantitative analyses of thymidylate synthase inhibition by N^4-OH-dCMP and its analogues, leading to time-dependent inactivation of the enzyme, were performed by following the decrease of enzyme activity with time (usually at 0.5, 1.0, 1.5, 4, 6, 8, and 10 min) during preincubation of the enzyme at 37 °C in the presence of 0.8 mM (±)-L-CH_2FH_4, 3.3 μM dUMP (to prevent thermal inactivation), and various concentrations of inhibitor. Activity remaining after preincubation was determined by addition of 25 μM [5-^{3}H]dUMP (7 ×10^4 dpm/nmol) and measurement of tritium release following 4-min incubation. The slopes of the semilog plots of percent remaining activity vs preincubation time, expressing apparent inactivation rate constants (k_{app}) and corresponding inhibitor concentrations ([I]), were then replotted as double-reciprocal plots, in accordance with the relationship of Brouillette et al. (1979):

$$\frac{1}{k_{app}} = \left(\frac{K_i[S]}{K_m k_2} + \frac{K_i}{k_2} \right) \frac{1}{[I]} + \frac{1}{k_2}$$

where k_2 is the inactivation rate constant. The values of k_2 and K_i were determined from the plot intercept and slope, respectively (Brouillette et al., 1979).
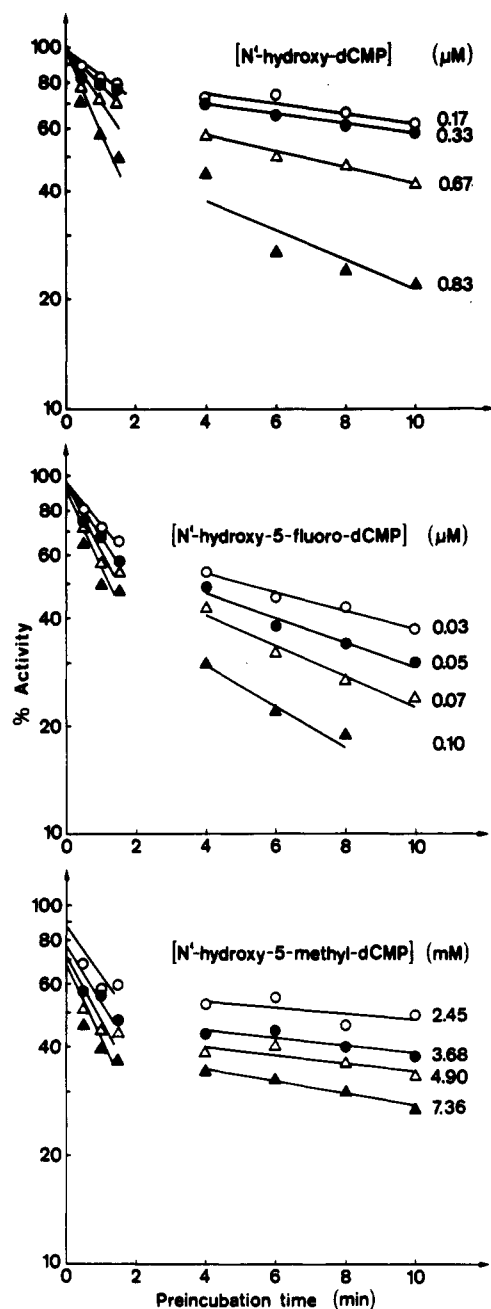
FIGURE 1: Slow-binding inhibition of Ehrlich carcinoma thymidylate synthase by N^4-hydroxy-dCMP (upper panel), N^4-hydroxy-5-fluoro-dCMP (middle panel), and N^4-hydroxy-5-methyl-dCMP (bottom panel).

Table II: Apparent K_i Values for Inhibition of Thymidylate Synthases from Ehrlich Carcinoma (E.c.), L1210 Parental (L1210) and 5-FdUrd-Resistant (L1210r) Cells, Regenerating Rat Liver (r.r.l.), and *H. diminuta* (H.d.) by dCMP, Its N^4-Substituted Analogues, and 5-FdUMP[a]

	K_i (μM)				
analogue	E.c.	L1210	L1210r	r.r.l.	H.d.
dCMP	12 000				
N^4-hydroxy-dCMP	4.1	1.7	9.3	2.6	1.0
N^4-hydroxy-5-fluoro-dCMP	2.6	0.8	1.4	0.3	0.2
N^4-hydroxy-5-methyl-dCMP	1 800				
N^4-methoxy-dCMP	380				
N^4-methoxy-5-fluoro-dCMP	22				
N^4-methoxy-5-methyl-dCMP	11 000				
5-FdUMP	0.01[b]	0.02	0.03	0.2[c]	0.01[c]

[a] Each analogue was added to the reaction mixture simultaneously with the substrate and cofactor. [b] Jastreboff et al., 1983. [c] Cieśla et al., 1990.
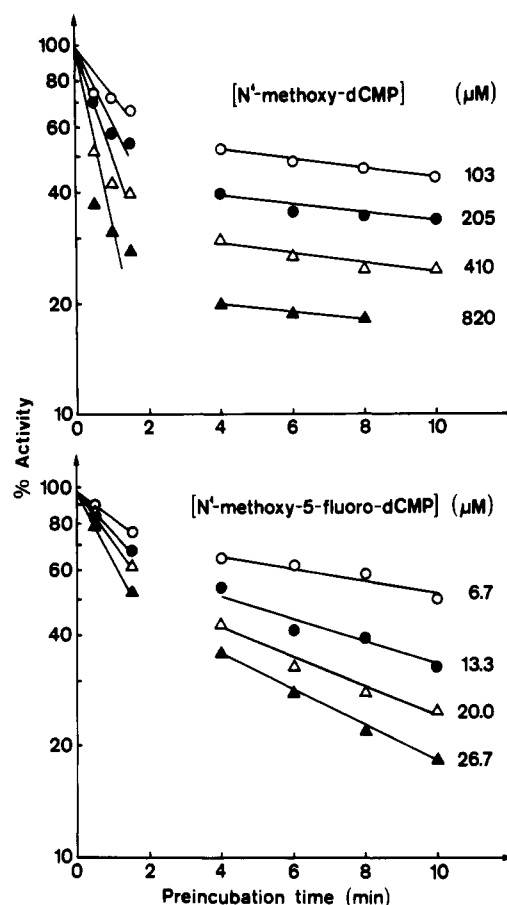

FIGURE 2: Slow-binding inhibition of Ehrlich carcinoma thymidylate synthase by N^4-methoxy-dCMP (upper panel) and N^4-methoxy-5-fluoro-dCMP (lower panel).

RESULTS

Inhibition of the Ehrlich carcinoma enzyme, by competition vs dUMP, of N^4-OH-dCMP and its analogues was examined by varying the dUMP concentration with different concentrations of inhibitor, added simultaneously to the reaction mixture. All compounds tested exhibited competitive inhibition, reflected by intersection at the ordinate of a Lineweaver–Burk plot (not shown). Similar patterns of competitive inhibition were observed for N^4-OH-dCMP and N^4-OH-FdCMP with the enzymes from parental and FdUrd-resistant L1210 cells, regenerating rat liver, and *H. diminuta*. The resultant apparent inhibition constants are listed in Table II. It should be noted that, for all five enzymes, the K_i values for N^4-OH-dCMP are further decreased on addition of a 5-fluoro substituent, the resulting enhancement of affinity varying appreciably between the different enzymes. By contrast, the K_i values for FdUMP are essentially the same. Furthermore,

with the *H. diminuta* enzyme, the K_i for N^4-OH-dCMP is only 1 order of magnitude higher than that for FdUMP.

Slow-Binding Inhibition. All the analogues, as well as dCMP, when preincubated with Ehrlich ascites thymidylate synthase and CH_2FH_4, led to time-dependent inactivation of the enzyme (Figures 1 and 2; 0.5 mM dCMP, in the presence of 3 μM dUMP, caused 8, 24, and 26% inactivation after 1.5, 4, and 8 min, respectively; because of its very low affinity for the enzyme, the amount of N^4-OMe-5-methyl-dCMP available was insufficient for this test), consistent with the behavior of each as a slow-binding inhibitor (Morrison, 1982). When

Table III: Parameters for Inactivation of Ehrlich Carcinoma Thymidylate Synthase by N^4-Hydroxy-dCMP, Its Analogues, and 5-FdUMP

compd	K_i' (μM)	K_i'' (μM)	k_2' (min^{-1})	k_2'' (min^{-1})
N^4-hydroxy-dCMP[a]	0.05	0.25	0.17	0.04
N^4-hydroxy-5-fluoro-dCMP[a]	0.02	0.04	0.40	0.09
N^4-hydroxy-5-methyl-dCMP	770	1610	0.28	0.03
N^4-methoxy-dCMP[a]	102	43	0.46	0.03
N^4-methoxy-5-fluoro-dCMP[a]	8	13	0.42	0.15
5-FdUMP[b]	0.006	0.07	0.18	0.17

[a] Results are means of two or more experiments which did not differ by more than 30%. [b] Rode et al., 1987.

N^4-OH-dCMP (2 μM), N^4-OMe-dCMP (0.6 mM), N^4-OH-5-methyl-dCMP (1.5 mM), or dCMP (2.2 mM) was preincubated with the Ehrlich carcinoma enzyme for up to 10 min, in the absence of CH_2FH_4, there was no loss of activity. With N^4-OH-FdCMP (0.15 μM) and N^4-OMe-FdCMP (5 μM), as well as FdUMP (25 nM), only small losses of activity ($\leq$20%) were observed, indicating dependence of the mechanism of inactivation on the presence of the cofactor, and hence on the reaction mechanism.

The rate of tritium release from [5-^{3}H]dUMP, added after preincubation in the presence of CH_2FH_4 of the N^4-OH or N^4-OMe derivatives of dCMP or FdCMP with Ehrlich carcinoma thymidylate synthase, as well as of FdUMP, N^4-OH-dCMP, or N^4-OH-FdCMP with either of the L1210 thymidylate synthases, was constant with time for up to at least 4 min (the 5-methyl derivatives were not tested). This permitted a kinetic evaluation of the time-dependent inactivation.

The inactivation rate always decreased after about 2-min preincubation, reflected by a biphasic plot of log (remaining activity) vs time (Figures 1 and 2), suggesting differing interactions of each inhibitor with the two binding sites on the thymidylate synthase molecule. The same pattern of time-dependent inactivation was found with regenerating rat liver and *H. dimunuta* enzyme preparations preincubated with N^4-OH-dCMP or its 5-fluoro derivative and CH_2FH_4. Consequently, inhibition constants and inactivation rate constants were calculated with the use of apparent inactivation rate constants during the initial (0.0–1.5 min) and later (4–10 min) periods of preincubation with a given inhibitor at various concentrations. The corresponding inhibition constants and inactivation rate constants are then K_i' and k_2' and K_i'' and k_2'', respectively (see Tables III–V).

Initial inhibition constants with Ehrlich carcinoma and L1210 thymidylate synthases for N^4-OH-dCMP were in the range 10^{-8} M and increased with preincubation time (Tables III and IV). Two-fold lower K_i values described the interactions of these enzyme preparations, as well as that from FdUrd-resistant cells, with N^4-OH-FdCMP (Tables III and

IV). By contrast, the inhibition constant for inhibition of the initially inactivated site of the Ehrlich carcinoma enzyme by N^4-OMe-dCMP (in the range 10^{-5} M) was 1 order of magnitude higher than that for inhibition by N^4-OMe-FdCMP (Table III). N^4-OH-dCMP, only 2-fold weaker than its 5-fluoro congener as an inhibitor, was 10^3 times more potent than N^4-OMe-dCMP and 10^4 times more potent than N^4-OH-5-methyl-dCMP, suggesting simultaneous requirements for presence of the N^4-hydroxyl and absence of a bulky substituent at C(5) for strong interaction with the enzyme.

Inhibition constants describing inactivation by N^4-OH-dCMP of regenerating rat liver and *H. diminuta* thymidylate synthases, during the initial period of preincubation, were 1 order of magnitude higher than those found for the mouse tumor (both Ehrlich carcinoma and L1210) enzymes (Table V).

Effect of 5-Fluoro Substituent. Inhibition of thymidylate synthase by N^4-OH-dCMP was potentiated to different extents by introduction of a 5-fluoro substituent (Tables II–V). Distinct potentiation occurred with the enzyme from the FdUrd-resistant L1210 cells (Table IV). The latter enzyme, compared to the parent L1210 thymidylate synthase, showed a lower affinity of the initially inactivated site for FdUMP, accompanied by lower affinities of both sites for N^4-OH-dCMP, but unchanged affinities of both sites for N^4-OH-FdCMP (Table IV). Similar potentiation of inhibition by N^4-OH-dCMP, following 5-fluoro substitution, was seen with the regenerating rat liver and *H. diminuta* thymidylate synthases (Tables II and V). On the other hand, with the enzyme from Ehrlich carcinoma and parental L1210 cells, the effect was rather weak (Tables II–IV).

Inactivation rate constants for the derivatives tested, including FdUMP, were quite similar, at least for the initially inactivated site (Tables III–V), pointing to a common inactivation rate-limiting step. Higher values were observed only for inactivation of the tapeworm enzyme by both 5-fluoro derivatives (Table V).

Test for Substrate Activity. To test whether thymidylate synthase may methylate N^4-OH-dCMP, the latter was substituted for dUMP in the enzyme reaction and absorbance at 338 nm monitored. When 0.35 mM N^4-OH-dCMP was incubated at 37 °C with the Ehrlich carcinoma enzyme (activity 0.5 nmol/min at pH 7 and 0.3 nmol/min at pH 9), in the presence of 0.36 mM ($\pm$)-L-CH_2FH_4, at pH 7 or 9, no absorbance change was observed for up to 30 min. This argues against 5-methylation of N^4-OH-dCMP [cf. Goldstein et al. (1984)], since this process is associated with formation of dihydrofolate, which should result in an increase of absorbance at 338 nm (Wahba & Friedkin, 1961).

Also tested was the possibility that thymidylate synthase, in the presence of CH_2FH_4, might liberate the C(5) proton

Table IV: Parameters for Inactivation by 5-FdUMP, N^4-Hydroxy-dCMP, and N^4-Hydroxy-5-fluoro-dCMP of Thymidylate Synthase from Parental (P) and 5-FdUrd-Resistant (R) L1210 Cells

enzyme	K_i' (nM)	K_i'' (nM)	k_2' (min^{-1})	k_2'' (min^{-1})
		5-FdUMP		
P	1.8 $\pm$ 0.4 (6)[a]	20 $\pm$ 5 (4)	0.17 $\pm$ 0.02 (6)	0.12 $\pm$ 0.04 (5)
R	12.2 $\pm$ 1.4 (6)	14 $\pm$ 3 (4)	0.25 $\pm$ 0.04 (6)	0.06 $\pm$ 0.02 (4)
		N^4-Hydroxy-dCMP		
P	63 $\pm$ 9 (4)	226 $\pm$ 35 (5)	0.17 $\pm$ 0.05 (4)	0.02 $\pm$ 0.00 (5)
R	184 $\pm$ 61 (6)	1460 $\pm$ 330 (5)	0.20 $\pm$ 0.04 (6)	0.09 $\pm$ 0.02 (5)
		N^4-Hydroxy-5-fluoro-dCMP		
P	73 $\pm$ 13 (4)	71 $\pm$ 6 (7)	0.24 $\pm$ 0.04 (4)	0.07 $\pm$ 0.01 (7)
R	93 $\pm$ 18 (4)	56 $\pm$ 9 (7)	0.24 $\pm$ 0.03 (4)	0.06 $\pm$ 0.00 (7)

[a] Results are presented as means $\pm$ SEM, followed by the number of separate experiments in parentheses.

Table V: Parameters for Inactivation of Regenerating Rat Liver (r.r.l.) and *H. diminuta* (H.d.) Thymidylate Synthases by N^4-Hydroxy-dCMP, N^4-Hydroxy-5-fluoro-dCMP, and 5-FdUMP

compd	enzyme source	K_i' (μM)	K_i'' (μM)	k_2' (min^{-1})	k_2'' (min^{-1})
N^4-hydroxy-dCMP	r.r.l.	0.89	0.13	0.27	0.02
	H.d.	0.92	0.21	0.38	0.01
N^4-hydroxy-5-fluoro-dCMP[a]	r.r.l.	0.05	0.02	0.44	0.10
	H.d.	0.05	0.02	0.67	0.09
5-FdUMP[a]	r.r.l.	0.01	0.11	0.20	0.30
	H.d.	0.11	0.02	1.1	0.10

[a] Ciesla et al., 1990.

Scheme I: dUMP (R_2 = H) and FdUMP (R_2 = F)[a]

[a] Neutral forms (left) and monoanions with distribution of negative charge between O^2 and O^4. For dUMP, pK_a = 9.5, hence low proportion of monoanion at neutral pH; for FdUMP, pK_a = 7.8, hence appreciable proportion of monoanion at neutral pH. R_1 = 2'-deoxyribose 5'-phosphate.

of N^4-OH-dCMP. When 0.1 μM N^4-OH-[5-^{3}H]dCMP (3.3 × 10^{10} dpm/μmol) was incubated at 37 °C with the Ehrlich carcinoma enzyme (activity 0.08 nmol/min at pH 7.5 and 0.06 nmol/min at pH 9.5), in the presence of 0.63 mM (±)-L-CH$_2$FH$_4$, at pH 7.5 or 9.5, no release of tritium (measured as radioactivity not absorbed by charcoal under conditions of the routine enzyme assay) was detected for up to 24 h.
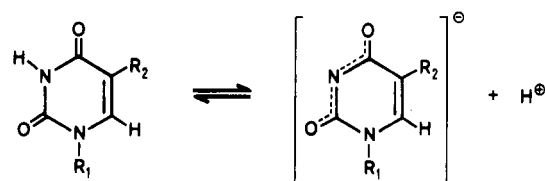
DISCUSSION

The foregoing results demonstrate that N^4-OH-dCMP, although not a substrate, is a competitive inhibitor, relative to dUMP, of murine tumor, as well as of regenerating rat liver and *H. diminuta* thymidylate synthases. In the presence of CH$_2$FH$_4$ it also produces time-dependent inactivation of the enzyme, as previously noted for the enzyme from chick embryo (Lorenson et al., 1967) and *Lactobacillus casei* (Galivan et al., 1976; Goldstein et al., 1984). In fact the K_i values for the analogue, from double-reciprocal plots, are comparable for all the enzymes (1–8 μM). The biphasic pattern of inactivation, indicating asymmetry of two binding sites, is in agreement with observations of Goldstein et al. (1984).

Previous attempts to evaluate kinetically the time-dependent inactivation of the enzyme (from a methotrexate-resistant strain of *Lactobacillus casei*) by N^4-OH-dCMP in the presence of CH$_2$FH$_4$ were unsuccessful, ascribed to apparent instability of the ternary complex following addition of dUMP to assay residual enzyme activity (Goldstein et al., 1984). Under our experimental conditions, all three of our murine thymidylate synthases formed ternary complexes which exhibited no lability on addition of dUMP, whether the inhibitor was N^4-OH-dCMP or, in those instances where employed, N^4-OMe-dCMP, or the corresponding 5-fluoro congeners. We also observed no indications of such lability with other thymidylate synthase preparations referred to below.

Kinetics of slow-binding inhibition of Ehrlich carcinoma and L1210 thymidylate synthases by N^4-OH-dCMP revealed it to be only 10-fold less potent than FdUMP, hence surprisingly effective (Tables III and IV). It was, on the other hand, 10-fold less effective against the enzyme from FdUrd-resistant L1210 cells (Table IV) and from normal tissues, viz., regenerating rat liver and *H. diminuta* (Table V).

By contrast, it was 1–2 orders of magnitude weaker than 5-FdUMP as a competitor of the substrate dUMP, with $K_{i\,app}$ values, from Lineweaver–Burk plots (Table II), in the range of reported K_m values for dUMP (1.3 μM, 2.6 μM, 2.5 μM, 5.3 μM, and 3.4 μM for the enzymes from Ehrlich carcinoma, parental and FdUrd-resistant L1210, regenerating rat liver, and *H. diminuta*, respectively; Jastreboff et al., 1983; Zieliński et al., 1990; Ciesla et al., 1990).

Somewhat unexpected was the finding that enhancement of the inhibitory effect of N^4-OH-dCMP (and N^4-OMe-dCMP) following introduction of a 5-fluoro substituent (2–20-fold) was lower than anticipated. The presence of the

5-fluorine enhanced inhibition parameters reflecting both time-dependent (K_i and k_2; Tables III–V) and time-independent ($K_{i\,app}$; Table II) enzyme–inhibitor interactions, suggesting that the affinity of the analogue for the enzyme, rather than the mechanism of inhibition, has changed (see also below).

The latter result suggested that mechanisms by which the 4-(hydroxyamino) and 5-fluoro substituents block the enzyme reaction may be mutually exclusive.

Inhibition by FdUMP is ascribed to the fact that, following formation of a ternary complex with enzyme and cofactor, the reaction terminates because the fluorine atom cannot be readily abstracted, as occurs with the dUMP C(5) hydrogen in the course of the normal thymidylate synthase reaction (Santi & Danenberg, 1984). With N^4-OH-dCMP, which forms a ternary complex and contains a hydrogen at C(5), a corresponding inhibition mechanism would have to result from an arrest of either liberation of the C(5) hydrogen in the enzyme–inhibitor–cofactor complex or some reaction step closely preceding the latter. Assuming such a mechanism for N^4-OH-dCMP, introduction of the 5-fluoro substituent should not drastically influence inhibition.

Results of the experiments with N^4-OH-[5-^{3}H]dCMP confirm lack of release of the C(5) proton from N^4-OH-dCMP, in the presence of CH$_2$FH$_4$, by Ehrlich carcinoma thymidylate synthase. However, they do not allow us to conclude whether the latter phenomenon is the cause of the reaction arrest or the result of an arrest of some step prior to hydrogen abstraction and posterior to CH$_2$FH$_4$ addition to the pyrimidine ring. The former would have to be a consequence of a lower acidity of the proton at C(5) in N^4-OH-dCMP than in dUMP, while the latter could possibly result from some relatively strong interactions (hydrogen bonds?) between the N^4-OH group and the cofactor.

We are not aware of any data on the C(5) hydrogen acidity in N^4-OH-dCMP. On the other hand, considering the involvement of N^4-OH-dCMP in the stereochemical path for thymidylate synthase catalysis, proposed by Slieker and Benkovic (1984), we prepared a stick model of the ternary complex of the enzyme with the cofactor and inhibitor, which pointed to possible hydrogen bonding between the imino N^4-hydroxyl oxygen and N^{10} of CH$_2$FH$_4$.

Physicochemical Properties of Inhibitors. A better appreciation of the foregoing experimental data requires some consideration of the structure and properties of the inhibitors embraced in this study.

The 5-fluorouracil ring in FdUMP, the neutral form of which is 2,4-diketo, exhibits a pK_a for dissociation of the ring N(3)–H of 7.8, as compared to 9.6 for the uracil ring in dUMP. Hence, whereas dUMP (and dTMP) is in the neutral form at physiological pH, FdUMP under these conditions is a mixture of neutral and monoanionic species, the latter with a distribution of charge between two fluorouracil oxygens, as

Scheme II: Amino–Imino and Syn–Anti Equilibria for N^4-Hydroxy and N^4-Methoxy Derivatives of dCMP[a]
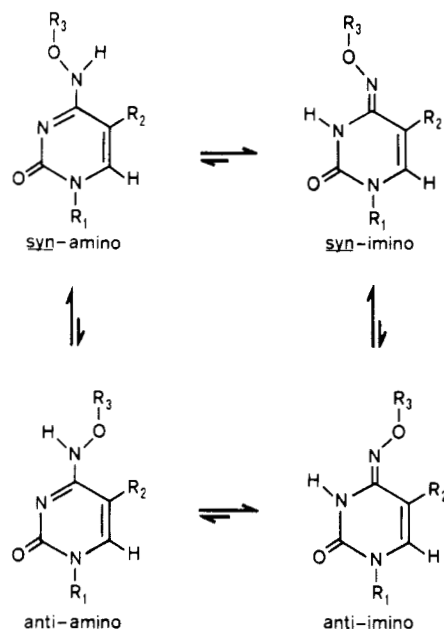
[a] N^4-Hydroxy-dCMP: R_2 = H; R_3 = H. N^4-Methoxy-dCMP: R_2 = H; R_3 = Me. N^4-Hydroxy-FdCMP: R_2 = F; R_3 = H. N^4-Methoxy-FdCMP: R_2 = F; R_3 = Me. N^4-Hydroxy-5-methyl-dCMP: R_2 = Me; R_3 = H. N^4-Methoxy-5-methyl-dCMP: R_2 = Me; R_3 = Me. R_1 = 2'-deoxyribose 5'-phosphate.

in Scheme I (Berens & Shugar, 1963; Wierzchowski et al., 1965). The extent to which the monoanionic form of FdUMP participates, if at all, in the inhibitory activity of FdUMP has not been adequately characterized. Examination of the pH dependence of inhibition by FdUMP and 5-fluoro-4-thio-dUMP (using the corresponding nucleosides relative to the nucleotides) pointed to the neutral forms as the more active species (Dzik et al., 1987), but some involvement of the monoanionic forms could not be unequivocally excluded.

The pK_a of N^4-OH-dCMP, for protonation of the N^4-hydroxycytosine ring, is 2.5; for its 5-fluoro analogue this is further reduced to 0.7 (see above). In alkaline medium these compounds also dissociate to form monoanions. For N^4-OH-dCMP the pK_a for such monoanion formation is 10.5 and corresponds to dissociation of the N(3)–H of the oximino form of the compound (Morozow et al., 1983). We have spectro-photometrically titrated N^4-OH-FdCMP to find a pK_a of 10.4 for monoanion formation. Hence, both compounds are uniquely in the neutral forms at physiological pH. Furthermore, for both of them, dissociation of the N(3)–H on monoanion formation is consistent with their being predominantly in the imino forms (see below).

It has been implicitly assumed [e.g., Fox et al. (1959) and Goldstein et al. (1984)] that N^4-OH-Cyd and N^4-OH-dCMP are in the amino form [but cf. Santi (1980)]. However, 1-substituted N^4-OH- and N^4-OMe-cytosines exhibit appreciable amino–imino tautomerism (see Scheme II) and have been shown to be predominantly in the imino form in polar media (Brown et al., 1967; Kierdaszuk & Shugar, 1983; Kierdaszuk et al., 1983). It is this form which is observed in the crystal (Shugar et al., 1976; Birnbaum et al., 1979), and it is pre-dominant in aqueous medium, to the extent of about 90% (Brown et al., 1967).

Furthermore, the exocyclic N^4-OH, or N^4-OMe, exists as an equilibrium mixture of two rotameric forms about the C(4)–N^4 bond, syn or anti relative to the ring N(3) (see Scheme II). In the solid state it is the *syn*-imino rotamer which

is observed (Shugar et al., 1976). In solution, where a syn–anti equilibrium exists, introduction of a bulky (e.g., CH_3) sub-stituent at C(5) sterically hinders formation of the anti ro-tamer, thus shifting the equilibrium strongly in favor of the syn form.

Thus, both N^4-OH- and N^4-OMe-dCMP are predominantly in the *syn*-imino form in aqueous medium. But the amino–imino equilibrium may be shifted appreciably by preferential base pairing of one or the other form (Stolarski et al., 1987); such a shift in the tautomeric equilibrium may also result from interaction with the enzyme.

Comparison of the inhibition constants of the various ana-logues, reflecting the level of competition with dUMP (Table II), underlines the role of the N^4-OH group. N^4-OH-dCMP is more potent than dCMP by 4 orders of magnitude. Fur-thermore, when the hydroxy group is replaced by methoxy, the inhibitory activity of the resulting N^4-methoxy-dCMP is 2 orders of magnitude lower than that of N^4-hydroxy-dCMP.

Effect of a 5-Methyl Substituent. Methylation of dUMP at C(5) reduces interaction with the enzyme by only 5-fold, since K_m for dUMP is 1.3 μM and K_i for dTMP is 7.3 μM with the Ehrlich carcinoma enzyme (Rode et al., 1984), with similar values for the enzyme from other sources (Santi & Danenberg, 1984). By contrast, the 5-methyl congener of N^4-hydroxy-dCMP is 10^4-fold less effective as a slow-binding inhibitor and 10^3-fold less potent as a competitor of dUMP, while the 5-methyl analogue of N^4-methoxy dCMP is 10^2-fold less active than the latter (Table II). Since the presence of a 5-methyl sterically constrains the N^4-OH (or OMe) group to the syn conformation (Shugar et al., 1976), it follows that the anti rotamers of N^4-hydroxy- and N^4-methoxy-dCMP are the active species which interact with the enzyme.

The foregoing is consistent with the observed inactivation of the enzyme from the parental and FdUrd-resistant L1210 cells by N^4-OH-dCMP. The enzyme from both sources ex-hibited similar interactions with dUMP, K_m values being 2.5 μM and 2.5 μM, respectively (Zieliński et al., 1990). But the enzyme from resistant cells was much less susceptible to slow-binding inhibition by both FdUMP and N^4-OH-dCMP (Table IV). Hence, an alteration affecting interaction with a 5-fluoro substituent correspondingly modified the interaction with an N^4-hydroxy substituent, a result to be expected if the N^4-hydroxyl is directed toward C(5) and forms a hydrogen bond with an amino acid residue in the close vicinity of the site of interaction of the 5-fluoro substituent.

Role of 5-Fluoro Substituent. Furthermore, assuming anti-N^4-OH-dCMP as the active inhibitor, and considering results presented in Table IV, it is possible to suggest an explanation for the influence of the 5-fluoro substituent in N^4-OH-dCMP (and N^4-OMe-dCMP) on the inhibitory po-tency. The finding that thymidylate synthase from FdUrd-resistant cells, as compared with that from parental L1210 cells, is less sensitive to inhibition by both FdUMP and N^4-OH-dCMP, but not by N^4-OH-FdCMP, suggests some in-terplay between the N^4-OH and C(5)–F groups in their in-teraction with the enzyme. Such an interplay might result from interaction of the two groups via hydrogen bonding, influencing the equilibrium between the rotameric forms by stabilization of the anti rotamer. Particularly relevant, in this context, is the fact that, the weaker the inhibitory effect of a given analogue, the more pronounced is the enhancement of affinity for the enzyme on introduction of a 5-fluoro sub-stituent.

Intra- and intermolecular interactions of the C–F bond in the biochemistry of fluorine-containing compounds have

aroused considerable interest, and some controversy. For example, the electron-withdrawing ability of fluorine is such as to cause sodium fluoropyruvate to exist mainly as the *gem*-diol, rather than the carbonyl, form, even in aqueous medium (Goldstein et al., 1978; Hurley et al., 1979). Experimental and theoretical evidence for involvement of the C–F bond in interactions with other groups, including C–F⋯H(O,N) hydrogen bonding, has been concisely reviewed by Murray-Rust et al. (1983).

Therefore, the C(5)–F group should be included in analyses of thymidylate synthase inhibition not only as the cause of reaction termination, resulting from the strength of the C–F bond, but also as a potential hydrogen acceptor in intra- or intermolecular hydrogen bonding which may influence the structure and stability of the enzyme–inhibitory–cofactor ternary complex.

Particularly interesting is the apparent specificity exhibited by N^4-OH-dCMP, reflected in its more potent inhibition of the enzyme from tumor (Ehrlich carcinoma and L1210; Tables III and IV) as compared to normal (regenerating rat liver and *H. diminuta*; Table V) tissues.

One consequence of the finding that *anti*-N^4-OH-dCMP is the apparent active inhibitor is that slow-binding inhibition by this rotamer, present in the free form in solution to the extent of only 5% (Shugar et al., 1976), is probably as effective, at least with the mouse tumor enzymes, as FdUMP. Of obvious interest would be an analogue exclusively in the anti form. N^4-OH-FdCMP, which is as potent as FdUMP vs the *H. diminuta* enzyme (Table V), appears to be a prototype of such an analogue, with the anti rotamer stabilized by noncovalent intramolecular bonding.

Attempts to stabilize the anti rotamer are underway. Meanwhile, during completion of this paper, Lin and Brown (1989) reported the synthesis of an analogue of N^4-OH-dCyd in which the N^4 oxygen is linked to C(5) of the pyrimidine ring via two C–C bonds, so that it is in the fixed anti form. Although the spectral properties of this compound further confirmed predominance of the imino tautomer, it no longer contains the free N^4-hydroxyl nor the C(5) hydrogen. Nonetheless, a sample kindly provided by Dr. D. M. Brown was phosphorylated with the nucleoside phosphotransferase system and tested vs the Ehrlich carcinoma enzyme. It proved to be only a moderate inhibitor ($K_i \approx 0.04$ mM), indicating that further efforts to stabilize the anti rotamer must leave the N^4-hydroxy or/and the C(5)–H intact.

Studies of the FdUMP–CH$_2$FH$_4$–enzyme ternary complex [see Santi and Danenberg (1984) for review] have proven informative regarding the nature of the catalytic mechanism of thymidylate synthase. It clearly would be desirable to extend such physicochemical studies to ternary complexes in which FdUMP is replaced by N^4-OH-dCMP and its 5-fluoro derivative. Such studies would be expected to throw further light on the stereochemistry of the reaction, since (see above) the N^4-OH-dCMP–CH$_2$FH$_4$–enzyme complex may be the analogue of a steady-state intermediate of the reaction arrested at some step prior to (and hence different from the ternary complex with FdUMP) hydrogen abstraction at C(5).

ACKNOWLEDGMENTS

Registry No. N^4-Hydroxy-dCMP, 2277-58-9; N^4-hydroxy-5-fluoro-dCMP, 114333-22-1; N^4-hydroxy-5-methyl-dCMP, 129966-50-3; N^4-methoxy-dCMP, 91886-20-3; N^4-methoxy-5-fluoro-dCMP, 129966-49-0; N^4-methoxy-5-methyl-dCMP, 129966-51-4; 5-FdUMP, 134-46-3; 2'-deoxy-4-thio-5-FUMP, 114319-04-9; thymidylate synthase, 9031-61-2.

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Coupled Responses of the Regions Near Cysteine-190 and the Carboxy Terminus of Rabbit Cardiac Tropomyosin: Fluorescence and Circular Dichroism Studies[†]

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Received June 7, 1990; Revised Manuscript Received August 17, 1990

ABSTRACT: Rabbit cardiac tropomyosin was labeled at Cys-190 with either N-(1-pyrenyl)iodoacetamide (Py) or 6-acryloyl-2-(dimethylamino)naphthalene (AD, acrylodan). Half of the labeled sample then was treated with carboxypeptidase A to produce an identically labeled nonpolymerizable form of tropomyosin, NPTM. Investigation of temperature-dependent changes in pyrene excimer emission, acrylodan fluorescence polarization, and tyrosyl circular dichroism in different samples of tropomyosin and NPTM reveals that absence of the COOH-terminal portion of tropomyosin modifies the response of the Cys-190 region to heat. Removal of the COOH terminus releases certain conformational constraints from the coiled-coil back to and including the Cys-190 region without causing a severe drop in the net α-helical content of the protein. Observation of changes in pyrene excimer fluorescence and in fluorescence polarization of acrylodan with time after addition of carboxypeptidase A to samples of labeled tropomyosin directly demonstrates this relaxation process. Thermally induced reduction in tyrosyl circular dichroism, together with consideration of the distribution of tyrosyl residues on tropomyosin, also supports the proposal.

Tropomyosin (TM)[1] is a rodlike molecule composed of two parallel α-helical polypeptide chains wrapped around each other to form a coiled coil [reviewed by Smillie (1979), Côté (1983), and Marston and Smith (1985)]. TM from rabbit cardiac muscle consists of 2 identical chains, each 284 residues long and each containing a single cysteine residue at position 190 (Lewis & Smillie, 1980). In skeletal and cardiac muscles, TM lies in a head-to-tail manner along the grooves of F-actin filaments and works with troponin to confer Ca^{2+} sensitivity to muscle contraction [reviewed by McCubbin and Kay (1980)]. The end-to-end interactions of TM are linked to its function in the Ca^{2+}-dependent regulatory systems of striated muscle. They involve the overlap of eight or nine amino acids (Johnson & Smillie, 1975, 1977; Mclachlan & Stewart, 1975; Phillips et al., 1986) and are manifested in low ionic strength solutions in vitro by an increased solution viscosity relative to solutions at physiological or higher ionic strengths. This increase in viscosity can be abolished by removal of COOH-terminal amino acids by treatment with carboxypeptidase A to produce a nonpolymerizable form of TM (NPTM) (Ueno et al., 1976; Mak & Smillie, 1981a). NPTM alone does not bind to F-actin but can be induced to do so by the addition of whole troponin in the presence or absence of Ca^{2+} (Heeley et al., 1987). This ternary system in the absence of free Ca^{2+} produces myosin S1 ATPase inhibition comparable to that of

a system with TM instead of NPTM, but activation of S1 ATPase activity by addition of Ca^{2+} is much reduced in the NPTM-containing system (Heeley et al., 1989a).

TM fully dephosphorylated at Ser-283 exhibits a lower degree of polymerizability than fully phosphorylated TM (Heeley et al., 1989b). Polymerizability can also be disrupted by changes at positions well removed from the COOH terminus, e.g., interaction with deoxyribonuclease I (Payne et al., 1986; Clark & Burtnick, 1989) and chemical modification at Cys-190 (Graceffa & Lehrer, 1980; Burtnick & Racic, 1988). ^{1}H NMR studies of His-153 and His-276 of rabbit cardiac tropomyosin (Edwards & Sykes, 1980) suggest there to be a number of intermediate folded structures along the pathway of thermal denaturation for tropomyosin. Consistent with the idea of long-range communication along the tropomyosin coiled coil, carboxymethylation or cystine formation at Cys-190 alters the thermal dependence of the NMR signals from these His residues some 5.5 and 12.8 nm away, respectively.

In this study, we have attempted to increase our understanding of how events that occur near Cys-190 are communicated to the COOH terminus and vice versa by comparing fluorescence and circular dichroism (CD) responses of intact and carboxypeptidase-truncated TM molecules. One of the

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[1] Abbreviations: Py, N-(1-pyrenyl)iodoacetamide; acrylodan (AD), 6-acryloyl-2-(dimethylamino)naphthalene; TM, tropomyosin; NPTM, nonpolymerizable rabbit cardiac tropomyosin prepared by digestion with carboxypeptidase A; DTT, DL-dithiothreitol; Mops, 3-(N-morpholino)-propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl.