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## Kinetics and Thermodynamics of the Interaction of 5-Fluoro-2'-deoxyuridylate with Thymidylate Synthase<sup>†</sup>

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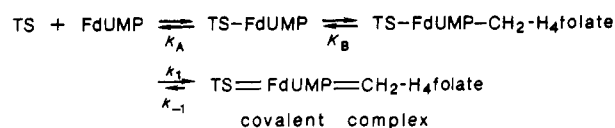
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**ABSTRACT:** Thymidylate synthase (TS), 5-fluorodeoxyuridylate (FdUMP), and 5,10-methylenetetrahydrofolate (CH<sub>2</sub>-H<sub>4</sub>folate) form a covalent complex in which a Cys thiol of TS is attached to the 6-position of FdUMP and the one-carbon unit of the cofactor is attached to the 5-position. The kinetics of formation of this covalent complex have been determined at several temperatures by semirapid quench methods. Together with previously reported data the results permit calculation of every rate and equilibrium constant in the interaction. Conversion of the noncovalent ternary complex to the corresponding covalent complex proceeds at a rate of 0.6 s<sup>-1</sup> at 25 °C, and the dissociation constant for loss of CH<sub>2</sub>-H<sub>4</sub>folate from the noncovalent ternary complex is ~1 μM. Activation parameters for the formation of the covalent complex were shown to be  $E_a = 20$  kcal/mol,  $\Delta G^\ddagger = 17.9$  kcal/mol,  $\Delta H^\ddagger = 19.3$  kcal/mol, and  $\Delta S^\ddagger = 0.005$  kcal/(mol-deg). The equilibrium constant between the noncovalent and covalent ternary complexes is  $\sim 2 \times 10^4$ , and the overall dissociation constant of CH<sub>2</sub>-H<sub>4</sub>folate from the covalent complex is  $\sim 10^{-11}$  M. The conversion of the noncovalent ternary complex to the covalent adduct is about 12-fold slower than  $k_{cat}$  in the normal enzymic reaction. However, because the dissociation constant for CH<sub>2</sub>-H<sub>4</sub>folate from the noncovalent ternary complex is about 10-fold lower than that from the TS-dUMP-CH<sub>2</sub>-H<sub>4</sub>folate Michaelis complex, the terms corresponding to  $k_{cat}/K_m$  are nearly equal. We propose that some of the intrinsic binding energy of CH<sub>2</sub>-H<sub>4</sub>folate may be used to facilitate formation of a 5-iminium ion intermediate. We suggest that iminium ion formation is catalyzed by (a) general-acid catalysis at N-10 of CH<sub>2</sub>-H<sub>4</sub>folate and (b) enzyme-induced perturbations of the five-membered ring of the cofactor within the noncovalent TS-FdUMP-CH<sub>2</sub>-H<sub>4</sub>folate complex. The latter may involve hydrogen bonding of the enzyme general-acid catalyst to N-10, perturbation of the *p*-aminobenzoic acid moiety of the cofactor, and strain on the five-membered ring of the cofactor.

5-Fluoro-2'-deoxyuridylate (FdUMP)<sup>1</sup> behaves as a mechanism-based inhibitor in the thymidylate synthase (TS) reaction. In the presence of TS, CH<sub>2</sub>-H<sub>4</sub>folate, and FdUMP, enzymic conversions occur up to the step normally associated with methyl transfer to the pyrimidine heterocycle, thereby trapping a covalent TS=FdUMP=CH<sub>2</sub>-H<sub>4</sub>folate complex. The covalent complex is an analogue of a steady-state intermediate of the normal enzymic reaction and has a Cys thiol of TS attached to the 6-position of FdUMP and the one-carbon

### Scheme I



unit of the cofactor attached to the 5-position of the nucleotide (Santi & Danenberg, 1984). Because of its importance in chemotherapy of neoplastic disease, the interaction of FdUMP with TS has been extensively studied. Furthermore, since the interaction mimics several steps in the normal enzymic reaction, it provides an approach toward studying individual steps of the reaction that are otherwise inaccessible. Now that the

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<sup>1</sup> Abbreviations: TS, thymidylate synthase; FdUMP, 5-fluoro-2'-deoxyuridylate; CH<sub>2</sub>-H<sub>4</sub>folate, (6R)-L-5,10-methylenetetrahydrofolate; TS-FdUMP-CH<sub>2</sub>-H<sub>4</sub>folate, noncovalent ternary complex; TS=FdUMP=CH<sub>2</sub>-H<sub>4</sub>folate, covalent ternary complex; NMM, *N*-methylmorpholine; DTT, dithiothreitol; PABA, *p*-aminobenzoic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid.

crystal structure of TS is known (Hardy et al., 1987), this capability has taken on new importance. Following is a summary of the current status of our knowledge of the interaction of TS with FdUMP and  $\text{CH}_2\text{-H}_4\text{folate}$ .

The interaction of TS, FdUMP, and  $\text{CH}_2\text{-H}_4\text{folate}$  (Scheme I) proceeds by an ordered mechanism (Danenberg & Danenberg, 1978). TS binds FdUMP first and then  $\text{CH}_2\text{-H}_4\text{folate}$  to form a rapidly reversible, noncovalent ternary complex; this complex then undergoes unimolecular conversion to the covalent  $\text{TS}=\text{FdUMP}=\text{CH}_2\text{-H}_4\text{folate}$  complex. The formation of the binary complex has been studied by several workers, and dissociation constants under a variety of conditions have been reported [see Lewis and Dunlap (1981), and Mittelstaedt and Schimerlik (1986)]. The rapid conversion of the noncovalent ternary complex to the covalent ternary complex has thus far prevented direct determination of the dissociation constant ( $K_B$ ) of  $\text{CH}_2\text{-H}_4\text{folate}$  from the noncovalent complex; it has been tacitly assumed that  $K_B$  is similar to  $K_m$  for  $\text{CH}_2\text{-H}_4\text{folate}$  in the normal enzymic reaction. Likewise, our knowledge of the kinetics and thermodynamics of the formation and disruption of the covalent  $\text{TS}=\text{FdUMP}=\text{CH}_2\text{-H}_4\text{folate}$  complex is incomplete. The dissociation of FdUMP from the covalent  $\text{TS}=\text{FdUMP}=\text{CH}_2\text{-H}_4\text{folate}$  complex at various temperatures has been studied in some detail (Santi et al., 1974a; Danenberg & Danenberg, 1978; Bruice & Santi, 1982). The kinetics of formation of the covalent complex have also been described but, because the conversion is so fast, are difficult to assess. Previously reported (Santi et al., 1974a; Danenberg & Danenberg, 1978) kinetics of formation were indirectly determined and superficially treated as a simple bimolecular reaction; they are not very informative at the level of detail desired. Finally, we do not have a good understanding of the contribution that covalent bond formation provides to the stability of the ternary complex.

In the present work, the rates of formation of the covalent  $\text{TS}=\text{FdUMP}=\text{CH}_2\text{-H}_4\text{folate}$  complex have been measured at various temperatures by semirapid kinetic methods. Experimental conditions were designed so that the reaction could be conveniently analyzed by pseudo-first-order inactivation kinetics, leaving no ambiguity in interpretation of the primary data. The data permit us to directly determine values for  $k_1$  and  $K_B$  which, together with results from other studies, permit us to deduce all of the remaining parameters for the interactions depicted in Scheme I.

#### EXPERIMENTAL PROCEDURES

**Materials.** TS from methotrexate-resistant *Lactobacillus casei* was the homogeneous preparation previously described (Santi et al., 1974a; Wataya & Santi, 1977). The preparation of FdUMP,  $[6\text{-}^3\text{H}]\text{FdUMP}$ , (6*RS*)-L- $\text{CH}_2\text{-H}_4\text{folate}$ , and (6*R*)-L- $\text{CH}_2\text{-H}_4\text{folate}$  have been described (Santi et al., 1974a; Bruice & Santi, 1982). DTT was purchased from P-L Biochemicals, and NMM (sequanal grade) was obtained from Pierce. General Biochemicals supplied the bovine serum albumin; the standard 1.00 M  $\text{MgCl}_2$  solution was purchased from Fisher. The water used in all experiments was doubly distilled and purged with  $\text{N}_2$ . All other materials used have been described (Santi et al., 1974a,b; Bruice & Santi, 1982) or were the purest commercial grade available.

$[6\text{-}^3\text{H}]\text{FdUMP}$  was purified and its specific activity was determined shortly before use. The  $\epsilon_{260}$  of FdUMP is  $8950 \text{ M}^{-1} \text{ cm}^{-1}$  at pH 2 and  $8300 \text{ M}^{-1} \text{ cm}^{-1}$  at pH 7.2 (Bruice & Santi, 1982). Total enzyme concentration was determined by using  $\epsilon_{278} = 1.07 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$  (Santi et al., 1974a). The concentration of TS binding sites was measured by active site titration with  $[6\text{-}^3\text{H}]\text{FdUMP}$  in the presence of  $\text{CH}_2\text{-H}_4\text{folate}$

with appropriate corrections for filtration efficiency (Santi et al., 1974b).

**NMM Buffer.** To approximately 175 mL of  $\text{H}_2\text{O}$  was added 3.79 g of NMM, 3.48 mg of bovine serum albumin, 18.75 mL of 1.00 M  $\text{MgCl}_2$ , and 7.5 mL of 0.1 M  $\text{Na}_2\text{EDTA}$ . The pH was adjusted to 7.45 with about 0.8 mL of concentrated HCl; care was taken not to allow the temperature of the solution to rise above  $25^\circ\text{C}$ . The volume was then increased to about 240 mL, the pH adjusted to 7.40 with HCl, and the volume brought to 250 mL. Fresh solutions were prepared at least every 3 months. The final concentrations of the components were 75 mM  $\text{MgCl}_2$ , 3 mM EDTA, and 150 mM NMM. To 102.4 mL of  $\text{H}_2\text{O}$  was added 72 mL of the aforementioned buffer and 1.2 mL of 1.5 M  $\text{H}_2\text{CO}$ . This solution was prepared fresh weekly. Final concentrations were 60.7 mM NMM, 30.8 mM  $\text{MgCl}_2$ , 1.2 mM EDTA, 10.1 mM  $\text{H}_2\text{CO}$ , and  $13.5 \mu\text{g/mL}$  bovine serum albumin.

**DTT Solution.** Ten milliliters of an aqueous solution containing 400 mg of DTT was prepared. A quantitative thiol determination was performed by using 5,5'-dithiobis(2-nitrobenzoic acid), and the final DTT concentration was adjusted to 225 mM after adjusting the pH to ca. 7.4 with 0.1 M KOH. This solution was stored under argon and was prepared fresh every 3 days.

**Kinetic Buffer.** The NMM buffer (22.3 mL) was equilibrated to the temperature of the desired kinetic run, and the pH was adjusted to 7.40 with 1 M KOH. KCl (1.0 M) was added to make the total volume of KCl plus KOH added 200  $\mu\text{L}$ , giving  $\mu = 0.04$ . DTT solution (4.5 mL, 225 mM) was added after pH adjustment to avoid errors incurred with many glass electrodes in the presence of thiols (Hill & Spivey, 1974). Final concentrations were 50 mM NMM, 8.3 mM  $\text{H}_2\text{CO}$ , 11.1  $\mu\text{g/mL}$  BSA, 7 mM KCl, and 37.5 mM DTT.

**Kinetic Experiments.** Kinetic buffer containing the appropriate concentrations of enzyme and (6*R*)-L- $\text{CH}_2\text{-H}_4\text{folate}$  was added to stoppered 3-mL plastic vials (up to eight per run) containing micro stirring bars. After at least 10-min equilibration in a constant temperature ( $\pm 0.1^\circ\text{C}$ ) water bath containing an immersible magnetic stirrer, the reaction was initiated by rapidly injecting a 25- $\mu\text{L}$  solution of radiolabeled FdUMP (ca. 1.5 pmol; 14 000 dpm) from a thermostated jacketed Hamilton syringe into the enzyme solution; the reaction was quenched by rapid injection of 50  $\mu\text{L}$  of 0.45 mM nonradioactive FdUMP (22.5 nmol; ca. 15 000-fold excess over radioactive FdUMP). The solution was rapidly stirred during injections; a clock switch was activated simultaneously with both injections. After quenching, the complex was stored in the dark on ice for about 1 h prior to filtration of 400  $\mu\text{L}$  through nitrocellulose membranes (Santi et al., 1974b). The loss of bound FdUMP from the complex prior to filtration was negligible at  $0^\circ\text{C}$  ( $t_{1/2} > 30 \text{ h}$ ). The aforementioned radioactive FdUMP solution (25  $\mu\text{L}$ ) was filtered through nitrocellulose filters to provide a blank. All other techniques used have been described (Santi et al., 1974a,b; Bruice & Santi, 1982).

#### RESULTS

The ordered interaction of FdUMP and  $\text{CH}_2\text{-H}_4\text{folate}$  with TS to form the covalent  $\text{TS}=\text{FdUMP}=\text{CH}_2\text{-H}_4\text{folate}$  complex as described in Scheme I serves as the model used in this work. By use of  $[6\text{-}^3\text{H}]\text{FdUMP}$ , the covalent  $\text{TS}=\text{FdUMP}=\text{CH}_2\text{-H}_4\text{folate}$  complex can be isolated free of unbound  $[6\text{-}^3\text{H}]\text{FdUMP}$  and noncovalently bound  $\text{TS}-[6\text{-}^3\text{H}]\text{FdUMP}$  complexes, and the formation of the covalent  $\text{TS}=\text{FdUMP}=\text{CH}_2\text{-H}_4\text{folate}$  complex can be measured over time. The rate of formation of the covalent complex is fast ( $t_{1/2} =$

2–20 s), so measurements were made by rapid initiation of reactions with  $[6\text{-}^3\text{H}]\text{FdUMP}$  and quenching with excess unlabeled FdUMP. Quenching with unlabeled ligand prevents reassociation of any  $[6\text{-}^3\text{H}]\text{FdUMP}$  released from the enzyme and thus makes dissociation essentially irreversible. The binary  $\text{TS}\text{--}[6\text{-}^3\text{H}]\text{FdUMP}$  complex is not trapped on nitrocellulose under the conditions used (Santi et al., 1974b), so it does not contribute to the filter-bound radioactivity in the present analysis. It is possible, perhaps probable, that noncovalent ternary complex present can bind to the filters and in theory could complicate our interpretations. However, as described later, the concentration of the noncovalent complex is so low compared to the covalent complex (ca.  $10^5$ -fold lower) that contribution of the former to filter-bound radioactivity can be ignored. Experiments were performed with limiting concentrations of  $[6\text{-}^3\text{H}]\text{FdUMP}$  and high concentrations of  $\text{CH}_2\text{--H}_4\text{folate}$  and TS. Conventionally, enzyme would have been used as the limiting component. The use of limiting FdUMP ensured that the complex formed had only one of the two sites of the dimeric TS occupied, thus avoiding potential problems which might arise from the putative negative cooperativity in this interaction (Galivan et al., 1976; Danenberg & Danenberg, 1979). Further, we found that the high concentrations of  $[6\text{-}^3\text{H}]\text{FdUMP}$  necessary to maintain the nucleotide under pseudo-first-order conditions gave unacceptably high background counts on the filters. Covalent complex formation is a well-behaved first-order process under the conditions used (Figure 1A). The reaction is linear with enzyme concentration up to at least 200 nM binding sites (data not shown).

Equation 1 describes the apparent first-order rate constants for formation of covalent  $\text{TS}\text{=FdUMP}\text{=CH}_2\text{--H}_4\text{folate}$  complex under conditions of limiting FdUMP, with excess

$$\frac{[\text{FdUMP}]_T}{V} = \frac{1}{k_{\text{obsd}}} = \left( \frac{K_A K_B}{k_1 [\text{TS}]} + \frac{K_B}{k_1} \right) \frac{1}{[\text{CH}_2\text{--H}_4\text{folate}]} + \frac{1}{k_1} \quad (1)$$

$\text{CH}_2\text{--H}_4\text{folate}$  and TS. Here,  $k_{\text{obsd}}$  is the pseudo-first-order rate constant for formation of the covalent  $\text{TS}\text{=FdUMP}\text{=CH}_2\text{--H}_4\text{folate}$  complex,  $K_A$  is the dissociation constant for the binary  $\text{TS}\text{--FdUMP}$  complex,  $K_B$  is the dissociation constant for  $\text{CH}_2\text{--H}_4\text{folate}$  from the reversible  $\text{TS}\text{--FdUMP}\text{--CH}_2\text{--H}_4\text{folate}$  complex, and  $k_1$  is the unimolecular rate constant for conversion of the reversible ternary complex to the covalent  $\text{TS}\text{=FdUMP}\text{=CH}_2\text{--H}_4\text{folate}$  complex. To derive eq 1, the rate of formation of the covalent ternary complex ( $V$ ) is set equal to  $k_1$  multiplied by the concentration of the noncovalent ternary complex ( $[\text{TS}\text{--FdUMP}\text{--CH}_2\text{--H}_4\text{folate}]$ ), which is expressed in terms of the total concentration of FdUMP ( $[\text{FdUMP}]_T$ ). Thus, the three terms in eq 1 refer to free FdUMP, to the binary complex, and to the noncovalent ternary complex. The assumptions made in the derivation are that the rate of dissociation of the covalent complex is insignificant under the conditions used and that formation and dissociation of reversible complexes are rapid compared to formation of the covalent adduct (i.e., reversible complexes are rapidly equilibrating). The former is valid since dissociation of the covalent complex is several orders of magnitude slower than its formation (Santi et al., 1974a; Danenberg & Danenberg, 1978); that the latter assumption is valid is shown by data described below and elsewhere (Bruce & Santi, 1982). Specifically, if the reaction of the  $\text{TS}\text{--FdUMP}$  complex with  $\text{CH}_2\text{--H}_4\text{folate}$  were rate limiting,  $k_{\text{obsd}}$  would be a linear function of  $\text{CH}_2\text{--H}_4\text{folate}$  but not show saturation at high

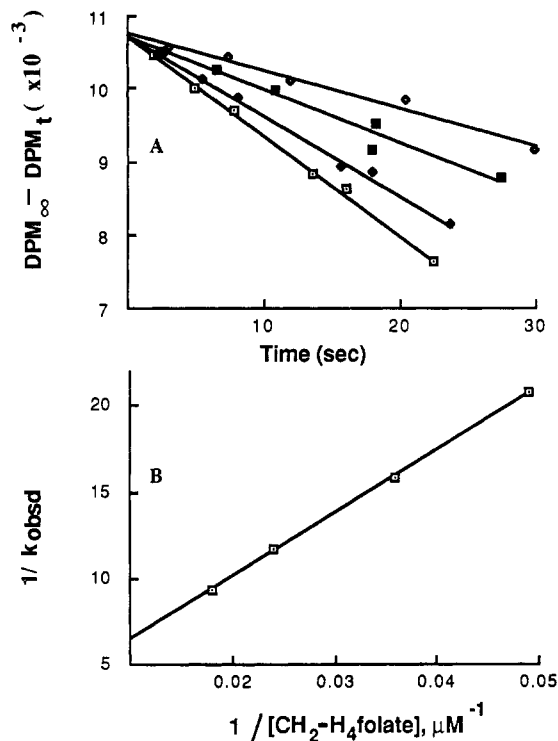


FIGURE 1: (A) First-order plots for appearance of filter-bound  $[6\text{-}^3\text{H}]\text{FdUMP}$  accompanying  $\text{TS}\text{=FdUMP}\text{=CH}_2\text{--H}_4\text{folate}$  complex formation at  $3.1^\circ\text{C}$  with varying  $(6R)\text{-L-CH}_2\text{--H}_4\text{folate}$  concentrations. Values were determined as the bound disintegrations per minute (dpm) upon completion of reaction ( $\text{DPM}_\infty$ ) minus the dpm ( $\text{DPM}_t$ ) at the time of assay. The lines are least-square fits to the points. Concentrations were 3.4 nM  $[6\text{-}^3\text{H}]\text{FdUMP}$ , 177 nM TS, and 12.1 ( $\diamond$ ), 18.2 ( $\blacksquare$ ), 30.3 ( $\circ$ ), and 48.5 ( $\square$ )  $\mu\text{M}$   $(6R)\text{-L-CH}_2\text{--H}_4\text{folate}$ . (B) Replot of the pseudo-first-order rate constants from panel A versus the concentration of  $(6R)\text{-L-CH}_2\text{--H}_4\text{folate}$ . The line was derived from the best fit to the nonlinear form of eq 1.

concentrations of the cofactor as predicted by eq 1 and shown here by experimentation. Also, the dissociation of  $[^3\text{H}]\text{CH}_2\text{--H}_4\text{folate}$  would not show a secondary kinetic isotope effect as it does (Bruce & Santi, 1982).

To obtain  $k_{\text{obsd}}$  for the formation of the covalent complex, at least five points were obtained at appropriate times for each experiment and plotted as shown in Figure 1A. For each of the temperatures,  $k_{\text{obsd}}$  values were obtained at four different  $\text{CH}_2\text{--H}_4\text{folate}$  concentrations ranging from about 12 to 50  $\mu\text{M}$ . The  $k_{\text{obsd}}$  values at each temperature were fitted to the nonlinear form of eq 1, and data were replotted as  $1/k_{\text{obsd}}$  versus  $1/[\text{CH}_2\text{--H}_4\text{folate}]$ . As shown in Figure 1B, plots of  $1/k_{\text{obsd}}$  versus  $1/[\text{CH}_2\text{--H}_4\text{folate}]$  are linear;  $k_1$  is obtained from the vertical intercept and corresponds to the rate constant at extrapolated infinite concentration of  $\text{CH}_2\text{--H}_4\text{folate}$ . Values for  $K_A$  at each of the temperatures were calculated by using the reported temperature dependence of  $K_A$  (Mittelstaedt & Schimerlik, 1986) adjusted to the  $K_A$  determined at  $30^\circ\text{C}$  (15  $\mu\text{M}$ ) under the conditions used in this study (Wataya et al., 1977; Bruce & Santi, 1982). With these values for  $k_1$  and  $K_A$  and the concentration of TS binding sites,  $K_B$  values were calculated from the slope at each temperature.

Table I gives values for  $k_1$  and  $K_B$  determined at several temperatures, as well as those extrapolated to  $25^\circ\text{C}$ . From the Arrhenius plot of these data (not shown), the energy of activation ( $E_a$ ) for formation of the covalent complex is calculated to be 19.9 kcal/mol; the van't Hoff plot indicates that  $K_B$  is not very temperature sensitive. Table I also shows (i) values of the rate constants for the dissociation of  $[6\text{-}^3\text{H}]\text{FdUMP}$  from the covalent complex<sup>2</sup> at specified temperatures

Table I: Thermodynamic and Kinetic Parameters for Interaction of FdUMP and CH<sub>2</sub>-H<sub>4</sub>folate with TS

temp (°C)	[FdUMP] (nM)	[TS] <sup>a</sup> (nM)	K <sub>A</sub> <sup>b</sup> (μM)	K <sub>B</sub> <sup>c</sup> (μM)	k <sub>1</sub> (s <sup>-1</sup> )	E <sub>a</sub> <sup>d</sup>	ΔG <sup>†</sup>	ΔH <sup>†</sup>	ΔS <sup>†</sup>	k <sub>-1</sub> <sup>e</sup> (10 <sup>-6</sup> s <sup>-1</sup> )	E <sub>a</sub>	ΔG <sup>†</sup>	ΔH <sup>†</sup>	ΔS <sup>†</sup>	k <sub>-1</sub> /k <sub>1</sub> (10 <sup>-5</sup> )	ΔG <sup>†</sup>
3.1	3.4	177	3.92	3.02	0.0369	19.86	17.92	19.31	0.005	0.616	29.0	23.95	28.45	0.016	1.67	6.03
7.1	2.3	134	4.83	8.75	0.0574		17.94	19.30	0.005	1.37		23.86	28.44	0.016	2.39	5.92
10.0	2.6	130	5.67	2.33	0.302		17.20	19.30	0.007	2.38		23.80	28.44	0.016	0.788	6.60
15.2	3.2	159	7.28	1.25	0.203		17.75	19.29	0.005	5.84		23.74	28.43	0.016	2.88	5.98
19.7	2.6	122	8.90	1.04	0.241		17.94	19.28	0.005	13.7		23.62	28.42	0.016	5.68	5.68
25.0			11.5		0.586		17.75	19.27	0.005	33.0		23.54	28.41	0.016	5.63	6.04

<sup>a</sup> TS concentration refers to total FdUMP binding sites as determined by active site titration. <sup>b</sup> K<sub>A</sub> values are extrapolated from K<sub>A</sub> = 15 μM at 30 °C [measured by Wataya et al. (1977) under the conditions used here] by using the temperature dependence of Mittelstaedt and Schimerlik (1986). <sup>c</sup> K<sub>B</sub> values are calculated from eq 1. <sup>d</sup> E<sub>a</sub>, ΔG<sup>†</sup>, and ΔH<sup>†</sup> values are in kcal/mol; ΔS<sup>†</sup> values are in kcal/(mol-deg). <sup>e</sup> k<sub>-1</sub> values are from Santi et al. (1974a).

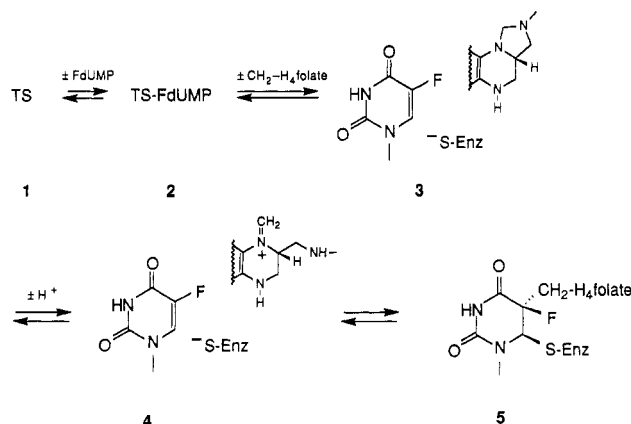


FIGURE 2: Minimal mechanism for formation of the TS=FdUMP=CH<sub>2</sub>-H<sub>4</sub>folate covalent complex. Free TS (1) interacts with FdUMP to give the binary complex 2 and then with CH<sub>2</sub>-H<sub>4</sub>folate to give the noncovalent ternary complex 3. Protonation of N-10 of the cofactor results in the enzyme-bound FdUMP-iminium ion 4. Formation of a covalent complex between the catalytic thiol of TS and C-6 of FdUMP, and between the one-carbon unit of the iminium ion and C-5 of FdUMP, results in the stable covalent complex 5.

(Santi et al., 1974a) which, without data to the contrary, may be assumed to reflect  $k_{-1}$ ; (ii) the equilibrium constants for the covalent versus noncovalent complex, determined as  $k_{-1}/k_1$ ; and (iii) the thermodynamic activation parameters for the formation and dissociation of the covalent complex.

## DISCUSSION

The composite of numerous studies has led to a good understanding of the minimal mechanism for the interaction of FdUMP, CH<sub>2</sub>-H<sub>4</sub>folate, and TS, which is summarized in Figure 2. The interaction of FdUMP (or dUMP), CH<sub>2</sub>-H<sub>4</sub>folate, and TS appears to proceed by an ordered mechanism with nucleotide binding first (Danenberg & Danenberg, 1978; Lu et al., 1984), at least under the conditions used here to study the reactions. Subsequent to formation of the reversible, noncovalent ternary complex, enzyme catalyzed conversions occur that result in formation of a covalent bond between C-6 of the nucleotide and the thiol of Cys-198 of TS and a covalent bond between C-5 of the nucleotide and the one-carbon unit (i.e., CH<sub>2</sub>-11) of the cofactor. The importance of this interaction to an understanding of catalysis is that it mimics several steps of the normal enzymic reaction, up to and including formation of the covalent adduct, which is a stable analogue of a steady-state intermediate. We may reasonably assume that the individual chemical conversions in this interaction

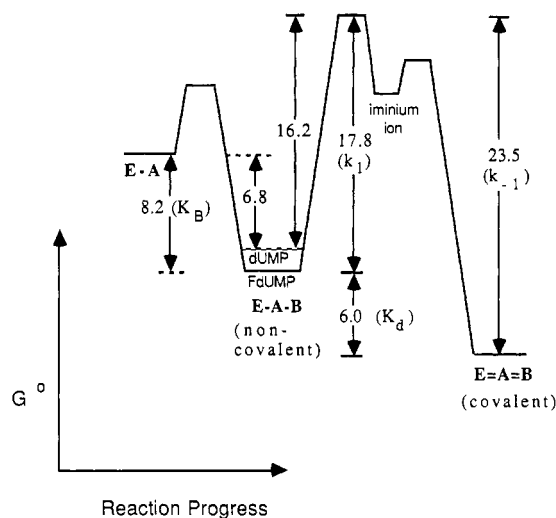


FIGURE 3: Reaction coordinate showing conversions of TS-FdUMP, TS-FdUMP-CH<sub>2</sub>-H<sub>4</sub>folate, and TS=FdUMP=CH<sub>2</sub>-H<sub>4</sub>folate complexes. ΔG<sub>B</sub>, ΔG<sub>C</sub> (the ΔG between the covalent and noncovalent ternary complexes), ΔG<sup>†</sup><sub>1</sub>, and ΔG<sup>†</sup><sub>-1</sub> are taken from data in Table I; ΔG<sup>†</sup><sub>B</sub> and the ΔG values associated with the 5-iminium ion are hypothetical.

proceed in a sequence analogous to the normal enzymic reaction. As such, the FdUMP interaction provides an entree to studies of individual steps of catalysis that are otherwise inaccessible.

Chemical models and other studies have led to the generally accepted conclusion that the reactive form of CH<sub>2</sub>-H<sub>4</sub>folate is the 5-iminium ion (Kallen & Jencks, 1966a; Benkovic, 1980) and that the reactive form of the nucleotide is a covalent dihydropyrimidine adduct (not shown) that possesses enol/enolate character at the C-5 position. Further, secondary α-hydrogen kinetic isotope studies of the FdUMP interaction have permitted the placement of the putative intermediates relative to the rate-determining step of the reaction (Bruice & Santi, 1982). The relevant points are as follows. (i) Covalent bond formation between the enzyme thiol and the C-6 of FdUMP occurs after the rate-determining step. (ii) Formation of the bond between C-5 of FdUMP and the one-carbon unit of the cofactor occurs during or after formation of the FdUMP covalent adduct. (iii) Formation of the 5-iminium ion precedes thiol addition at C-6 of FdUMP and probably occurs at the rate-determining step.

In the present work, we report experiments that led to determination of (i) the dissociation constants (K<sub>B</sub>) of CH<sub>2</sub>-H<sub>4</sub>folate from the noncovalent TS-FdUMP-CH<sub>2</sub>-H<sub>4</sub>folate complex and (ii) the rate constants for conversion of the noncovalent ternary complex to the covalent TS=FdUMP=CH<sub>2</sub>-H<sub>4</sub>folate complex. These constants represent the last such parameters remaining to be determined in this reaction and, together with previously reported data (see Table I), permit a complete kinetic and thermodynamic description of the interaction of FdUMP, CH<sub>2</sub>-H<sub>4</sub>folate, and TS. In addition,

<sup>2</sup> The rate constant measured for dissociation of [6-<sup>3</sup>H]FdUMP from the covalent complex has been reported to be  $3.33 \times 10^{-3} \text{ s}^{-1}$  at 25 °C; since there is a 23% α-tritium secondary isotope effect, this corresponds to  $4.1 \times 10^{-3} \text{ s}^{-1}$  for dissociation of the 6-proto compound. There is no isotope effect in formation of the complex, so the 1.23-fold difference in rate is also manifested in the dissociation constant. Values used here are the measured values for [6-<sup>3</sup>H]FdUMP.

these parameters have been determined at several temperatures, thereby allowing calculation of the corresponding activation parameters.

Figure 3 shows the free energy profile for the formation/disruption of the noncovalent/covalent FdUMP ternary complexes at 25 °C, using data listed in Table I. The energy of activation ( $E_a$ ) for covalent complex formation is 19.9 kcal/mol, with  $k_1$  values increasing about 3-fold per 10 °C. At 25 °C the rate constant for conversion of the noncovalent to the covalent ternary complex,  $k_1$ , is about 0.6 s<sup>-1</sup>;  $\Delta G^\ddagger$  is 17.8 kcal/mol with almost all of the contribution coming from  $\Delta H^\ddagger$  (19.3 kcal/mol). The reverse reaction is considerably slower than the forward with a rate constant of  $k_{-1} = 3.3 \times 10^{-5}$  s<sup>-1</sup> at 25 °C (Bruice & Santi, 1982; Santi et al., 1974a) and shows an even larger temperature coefficient ( $E_a = 29$  kcal/mol) with an ~11-fold increase per 10 °C;  $\Delta G^\ddagger$  is 23.5 kcal/mol at 25 °C and, as with  $k_1$ , has almost all its contribution from  $\Delta H^\ddagger$ . Thus, the equilibrium constant between the noncovalent and covalent complexes is also temperature dependent, showing a 2.2-fold increase per 10 °C. The dissociation constant of CH<sub>2</sub>-H<sub>4</sub>folate from the noncovalent complex is between 1 and 3  $\mu$ M over the temperature range examined; it is not very temperature dependent, showing a slight decrease of about 1.5-fold per 10 °C, which may be within our experimental error.

We now know the equilibrium constants relating every known species in the reaction. Dissociation constants of the binary TS-FdUMP complex have previously been reported (Mittelstaedt & Schimerlik, 1986). From the ratio  $k_{-1}/k_1$  determined here we can calculate the equilibrium constant between the noncovalent and covalent ternary complexes; at 25 °C this number ( $K_C$ ) is  $5.7 \times 10^{-5}$ , which corresponds to a  $\Delta G$  of -6.0 kcal/mol;  $K_B$  is about 1  $\mu$ M at 25 °C and shows little temperature dependence. The  $K_B$  is about 10-fold higher than the reported  $K_m$  values for CH<sub>2</sub>-H<sub>4</sub>folate in the normal enzymic reaction. Knowing  $K_B$  and  $k_{-1}/k_1$ , we calculate the overall (see Table I) equilibrium constant between the covalent TS=FdUMP=CH<sub>2</sub>-H<sub>4</sub>folate complex and the binary TS-FdUMP complex to be about  $5 \times 10^{-11}$  M at 25 °C. Workers have conventionally regarded FdUMP rather than CH<sub>2</sub>-H<sub>4</sub>folate as the "tight-binding" inhibitor of TS. In fact, this distinction cannot be made (Jencks, 1981). The extraordinarily low dissociation constant of the covalent complex formally defines the dissociation of CH<sub>2</sub>-H<sub>4</sub>folate from this complex, and CH<sub>2</sub>-H<sub>4</sub>folate does "induce" the formation of the tight complex. However, the ligands act with apparent synergism in forming the tight complex, and their individual contributions to stability of the complex are not currently known.

It is of interest to compare the interactions of FdUMP, CH<sub>2</sub>-H<sub>4</sub>folate, and TS to those of dUMP, CH<sub>2</sub>-H<sub>4</sub>folate, and TS in the normal catalytic reaction. In the conversion of dUMP to dTMP,  $k_{cat}$  is about 8 s<sup>-1</sup> at 25 °C (Pogolotti & Santi, 1979), which is some 13-fold greater than the corresponding  $k_1$  in the FdUMP reaction; this difference in rate constants represents a difference in  $\Delta G^\ddagger$  of 2.6 kcal/mol. However, the apparent dissociation constant of CH<sub>2</sub>-H<sub>4</sub>folate in the noncovalent TS-FdUMP-CH<sub>2</sub>-H<sub>4</sub>folate complex (1  $\mu$ M) is about 10-fold lower than in the analogous TS-dUMP-CH<sub>2</sub>-H<sub>4</sub>folate complex (10  $\mu$ M). Apparently, the affinity of the TS-FdUMP complex for CH<sub>2</sub>-H<sub>4</sub>folate is higher than that of the TS-dUMP complex. As a result of these compensatory differences, the  $k_1/K_B$  ( $6 \times 10^5$  M<sup>-1</sup> s<sup>-1</sup>) and  $k_{cat}/K_m$  ( $8 \times 10^5$  M<sup>-1</sup> s<sup>-1</sup>) values in the FdUMP and dUMP reactions, respectively, are nearly identical. One way to view this result is that the noncovalent TS-dUMP-CH<sub>2</sub>-H<sub>4</sub>folate

complex is positioned further along the reaction coordinate than the TS-FdUMP-CH<sub>2</sub>-H<sub>4</sub>folate complex. That is, some of the intrinsic binding energy of CH<sub>2</sub>-H<sub>4</sub>folate is used to facilitate catalysis. As described below, we speculate that some of the binding energy of the cofactor may be used to strain and/or increase the reactivity of the five-membered ring of CH<sub>2</sub>-H<sub>4</sub>folate, thus moving the cofactor closer toward the 5-iminium ion in the rate-determining step.

CH<sub>2</sub>-H<sub>4</sub>homofolate (an analogue of CH<sub>2</sub>-H<sub>4</sub>folate with an additional CH<sub>2</sub> between C-9 and N-10) has  $K_m$  and  $k_{cat}$  values which are 10- and 6-fold lower, respectively, than CH<sub>2</sub>-H<sub>4</sub>folate (Crusberg et al., 1970). Thus,  $k_{cat}/K_m$  for this analogue is also nearly identical with that of CH<sub>2</sub>-H<sub>4</sub>folate, which is again in accord with our proposal for facilitation of catalysis through the use of intrinsic binding energy of the cofactor. It is curious that there are also concomitant decreases in  $K_m$  and  $k_{cat}$  when the length of polyglutamates in CH<sub>2</sub>-H<sub>4</sub>folate is increased that keep  $V/K$  essentially constant (Lu et al., 1984); however, it has been pointed out that, at least with the TS from pig liver, the order of binding may change with increasing glutamate chain length, so  $V/K$  comparisons may be misleading (Lu et al., 1984; Matthews et al., 1987).

In the TS reaction, the reactants are relatively rigid and the enzyme is mobile. Several observations indicate a conformational change of the enzyme and significant perturbation of the environment of CH<sub>2</sub>-H<sub>4</sub>folate. As indicated by hydrodynamic, circular dichroism, and fluorescent quenching studies (Lewis & Dunlap, 1981; Lockshin et al., 1984), a large cofactor-dependent conformational change of the enzyme occurs upon formation of the TS-FdUMP-CH<sub>2</sub>-H<sub>4</sub>folate complex. A large absorbance peak at 320–340 nm also appears upon formation of the covalent TS=FdUMP=CH<sub>2</sub>-H<sub>4</sub>folate complex and of related ternary complexes containing nucleotide and/or cofactor analogues (Santi et al., 1974; Lewis & Dunlap, 1981), which is not present in the reactants and disappears upon SDS-PAGE denaturation of the complex. These properties seem to result from interactions of the protein with the PABA moiety of the cofactor and concomitant changes in their environment(s). Further, resonance Raman studies indicate that there is a significant change in the electronic structure of the PABA moiety in the ternary FdUMP complex (Fitzhugh et al., 1986), and it has been proposed that a negative charge (or H-bond acceptor) may reside near the 10-NH. Intriguingly, the crystal structure of TS shows an unusual cluster of seven aromatic amino acid side chains adjacent to the active site cavity that may be the site of this interaction (Hardy et al., 1987). Consistent with our proposal, the conformation of the native protein, as understood from the current crystal structure, does not accommodate binding of CH<sub>2</sub>-H<sub>4</sub>folate (unpublished data).<sup>3</sup>

*What Is the Nature of the Rate-Determining Step in the Formation of the Ternary Covalent FdUMP Complex?* Studies of secondary  $\alpha$ -hydrogen isotope effects using [<sup>11</sup>-<sup>3</sup>H]CH<sub>2</sub>-H<sub>4</sub>folate indicate that the conversion of 5,10-CH<sub>2</sub>-H<sub>4</sub>folate to the 5-iminium ion is the most likely candidate for the rate-determining step (Bruice & Santi, 1982). Chemical considerations have also led some workers to suggest that ring opening of CH<sub>2</sub>-H<sub>4</sub>folate might be a rate-limiting process in enzymic reactions (Fife et al., 1981; Benkovic, 1980). During

<sup>3</sup> Attempts to model binding of 5,10-CH<sub>2</sub>-H<sub>4</sub>folate to the reported (Hardy et al., 1987) three-dimensional structure of TS have thus far failed (unpublished data). It appears certain that conformational modifications of the protein structure will be necessary to model binding of the cofactor.

formation of the covalent complex (Figure 2), the  $sp^3$ -hybridized one-carbon unit of  $CH_2$ -H<sub>4</sub>folate (3) undergoes conversion to the  $sp^2$ -hybridized iminium ion (4), which then converts to the  $sp^3$ -hybridized covalent complex (5). Each of these two steps (i.e., 3 to 4 and 4 to 5) is calculated to proceed with a maximal (equilibrium) secondary  $\alpha$ -hydrogen kinetic isotope effect of *at least* 13% (probably greater), normal in the first step and inverse in the second.<sup>4,5</sup> If both conversions occurred before the rate-determining step, the effects would essentially cancel, while if both were post rate determining, neither would be observed. The interconversion of the covalent complex and the iminium ion is believed to occur at the same step as covalent bond formation to the 6-position of FdUMP, which has been shown *not* to be the rate-determining step (Bruice & Santi, 1982). It has been concluded that the formation of the covalent complex from  $CH_2$ -H<sub>4</sub>folate proceeds with a normal  $\alpha$ -hydrogen kinetic isotope effect at C-11 of about  $k_H/k_T \sim 1.08$ ,<sup>5</sup> which is significantly lower than the expected maximal effect and suggests that conversion of 5,10- $CH_2$ -H<sub>4</sub>folate to the 5-iminium ion occurs *during* the rate-determining step.

From chemical models, it is known that the 5-iminium ion is very unstable and rapidly reacts with nucleophiles or collapses back to 5,10- $CH_2$ -H<sub>4</sub>folate (Kallen & Jencks, 1966a). The formation of the 5-iminium ion requires protonation of N-10, but the reaction is fast and details of its catalysis are not well understood; nevertheless, features of the reaction can be deduced from chemical models and considerations.

**How Can TS Catalyze 5-Iminium Ion Formation?** Two reasonable general mechanisms can be envisioned for the TS-catalyzed conversion of the imidazolidine ring of 5,10- $CH_2$ -H<sub>4</sub>folate to the 5-iminium ion (Figure 4). The *first* involves general-acid-catalyzed cleavage of the N-10- $CH_2$ -11 bond, a mechanism suggested by previous workers (Fife et al., 1978, 1981; Benkovic, 1980). Of relevance here is the unsymmetrical  $pK_a$ 's of the 5- and 10-nitrogens of H<sub>4</sub>folate; the more basic is N-5 with  $pK_a$  4.85, which upon protonation leaves N-10 with a low  $pK_a$  of -1.25; the microscopic  $pK_a$  of the unperturbed N-10, as determined from models, is about 3

(Kallen & Jencks, 1966b). Proton donation to N-10 in the transition state would enhance its leaving group ability and weaken the N-10- $CH_2$ -11 bond, whereas electron release by N-5 would concomitantly stabilize the incipient iminium ion (Figure 4). A *second* general mechanism for this conversion involves enzyme-induced perturbations of the five-membered ring of the cofactor within the noncovalent TS-FdUMP- $CH_2$ -H<sub>4</sub>folate complex; that is, binding forces between the enzyme and cofactor could directly assist formation of the 5-iminium ion. (i) Hydrogen bond formation between the putative general-acid catalyst of the enzyme and N-10 in the ground state would increase its leaving group ability and probably cause some lengthening of the N-10-C-11 bond to be broken. (ii) There is evidence that the enzyme interacts favorably with the pteridine and/or PABA moieties of the cofactor, causing large perturbations in the environment of the PABA group and involving a conformational change of the enzyme.

Considerations of how the aforementioned enzyme-induced perturbations of the cofactor might effect iminium ion formation are presented here as a framework for further hypothesis and experimentation. (i) The proposed stress on the ground-state binding of  $CH_2$ -H<sub>4</sub>folate could be manifested by a rotation of the benzoyl part of the PABA moiety around the axis of the N-10 bond, disrupting the conjugation of the aromatic ring with the N-10. The consequence would be to make the N-10 more  $sp^3$ -like and hence more basic; this increased basicity would enhance hydrogen bond formation to the putative general acid, which would in turn increase reactivity. (ii) Electronic perturbations on the PABA moiety could themselves alter the chemical properties (e.g., basicity) of the N-10 and hence its leaving group potential. (iii) Finally, if enzyme-cofactor interactions are incompatible with the inherent constraints of the five-membered imidazolidine ring, there could be lengthening of the N-10-C-11 bond by strain. In this regard, studies of heterolytic  $S_N1$  C-O bond cleavages indicate that the ground-state lengths of bonds being broken correlate with their chemical reactivities (Jones & Kirby, 1984, 1986): "...the longer the bond, the faster it breaks"; also, "the more reactive the system, the more sensitive is the length (strength) to structural" perturbation (Edwards et al., 1986). Applied to  $CH_2$ -H<sub>4</sub>folate, a slight lengthening of the N-10-C-11 bond in the ground state (i.e., in a noncovalent complex) would render it more susceptible to cleavage<sup>6</sup> and more susceptible to perturbations of the environment of the leaving group (e.g., hydrogen bond formation at N-10 and/or enzyme-induced perturbation of the PABA moiety). These mechanisms are applications of theories on strain and distortion in enzyme catalysis (Jencks, 1969; Fersht, 1985).

Figure 4 summarizes salient features of our model. Here, the enzyme is conformationally mobile and the reactants are relatively rigid. The most stable state of the free enzyme (1) does not optimally fit  $CH_2$ -H<sub>4</sub>folate; binding of the nucleotide is required before the enzyme is accessible to the cofactor. We suggest that the binary enzyme-nucleotide complex (2) undergoes a conformational change toward 3, a structure more complementary to the transition state for iminium ion formation; hence, there is an apparent loss of intrinsic binding energy of  $CH_2$ -H<sub>4</sub>folate upon formation of the noncovalent ternary complex.<sup>3</sup> Within this complex (3), the enzyme interacts with  $CH_2$ -H<sub>4</sub>folate in a manner that alters the environment of the PABA group to increase the reactivity of the N-10-C-11 bond. This interaction could involve several

<sup>4</sup> Actually, the interconversion of 5,10- $CH_2$ -H<sub>4</sub>folate and the 5-iminium ion has been calculated to have a maximal equilibrium isotope effect of 1.05, with hydrogen concentrated in 5,10- $CH_2$ -H<sub>4</sub>folate (Bruice & Santi, 1982).

<sup>5</sup> The interpretations of  $\alpha$ -secondary tritium isotope effects in the interaction of FdUMP and  $CH_2$ -H<sub>4</sub>folate with TS have previously been described (Bruice & Santi, 1982). A brief summary follows. First, from kinetic isotope effect (KIE) studies of [6-<sup>3</sup>H]FdUMP we have concluded that conversion of 5 to 4 (the  $sp^2$ -hybridized iminium ion) is not rate limiting but rather pre rate determining in the *direction of dissociation*; upon dissociation, the KIE at C-6 of FdUMP is equal to both the theoretical and experimental equilibrium isotope effect (EIE) for interconversion of 5 and free FdUMP. Thus, in *formation* of the complex, conversion of 4 to 5 is post rate determining. Second, the small normal KIE observed in dissociation of [11-<sup>3</sup>H] $CH_2$ -H<sub>4</sub>folate [ $k_{obsd}(H/T) = 1.03$ ] indicates that 4 to  $CH_2$ -H<sub>4</sub>folate, and hence  $CH_2$ -H<sub>4</sub>folate to 4, is associated with the rate-determining step. The preequilibrium conversion of  $sp^3$ -hybridized [11-<sup>3</sup>H] $CH_2$ -H<sub>4</sub>folate of 5 to the  $sp^2$ -hybridized iminium ion must occur with a normal EIE ( $K_{5-4}$ ) which is  $\geq 1.13$  (a minimal value for the  $sp^3$  to  $sp^2$  conversion) and conversion of  $sp^2$ -hybridized 4 to  $sp^3$ -hybridized  $CH_2$ -H<sub>4</sub>folate must proceed with an inverse EIE of similar magnitude (Cleland, 1980). Since the dissociation of [11-<sup>3</sup>H] $CH_2$ -H<sub>4</sub>folate actually shows a net normal KIE of  $k_{obsd}(H/T) = 1.03$ , conversion of 4 to  $CH_2$ -H<sub>4</sub>folate must proceed with a less than maximal inverse KIE which incompletely cancels the normal EIE associated with 5 to 4. Using the EIE for conversion of 5 to 4, estimated from fractionation factors (Cleland, 1980), and the observed net KIE for dissociation, we calculate that conversion of 4 to  $CH_2$ -H<sub>4</sub>folate would occur with a KIE of  $k_{4-3}(H/T) = k_{obsd}(H/T)/K_{5-4}$ . Finally, in *formation* of the covalent complex we calculate the apparent KIE for conversion of  $CH_2$ -H<sub>4</sub>folate to the iminium ion (3-4) to be  $k_{obsd}(H/T)/0.95 = 1.08$ .

<sup>6</sup> In the acetal systems studied, bond length-reactivity plots showed 19-25 kcal mol<sup>-1</sup> Å<sup>-1</sup> (Edwards et al., 1986; Jones & Kirby, 1984).



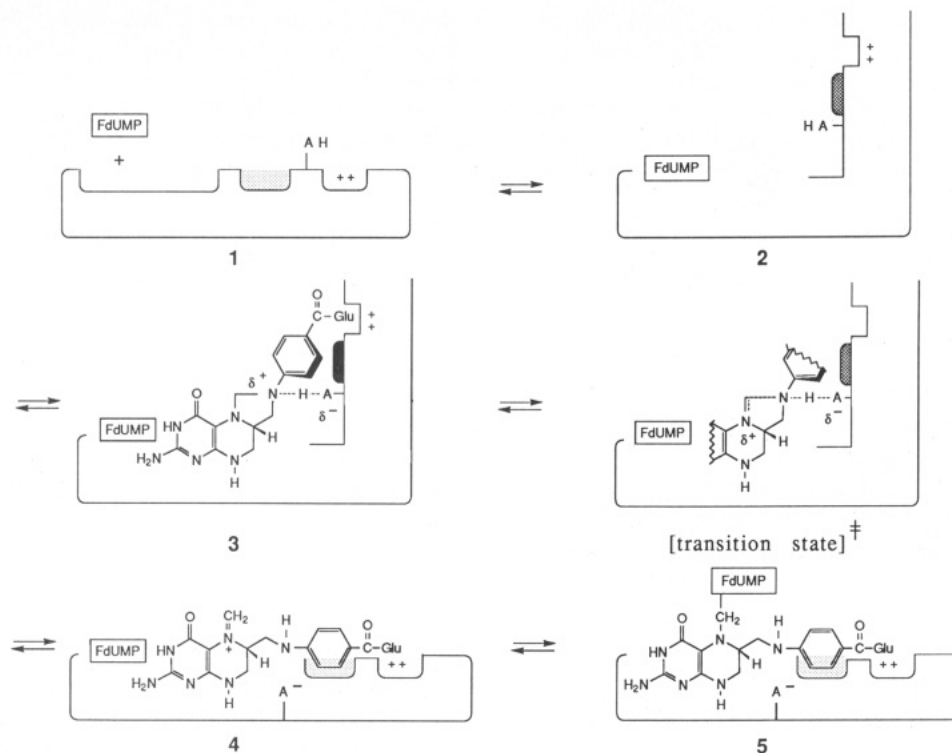


FIGURE 4: Proposed strain mechanism for conversion of  $\text{CH}_2\text{-H}_4\text{folate}$  to the 5-iminium ion in the TS-FdUMP(dUMP)- $\text{CH}_2\text{-H}_4\text{folate}$  complex. A-H represents a proposed general-acid catalyst of the enzyme which binds to N-10 and assists in lengthening and cleavage of the N-10- $\text{CH}_2\text{-11}$  bond of  $\text{CH}_2\text{-H}_4\text{folate}$ . The shaded area depicts a region of the enzyme with affinity for the PABA moiety of  $\text{CH}_2\text{-H}_4\text{folate}$ . **1** shows the stable form of the free enzyme which upon binding to FdUMP undergoes a conformational change to give the form of the enzyme (**2**) that can bind the cofactor. In the ternary complex (**3**) the N-10- $\text{CH}_2\text{-11}$  bond is lengthened and weakened; the transition state is depicted for conversion of the imidazolidine ring of the cofactor to the 5-iminium ion; **4** represents the bound 5-iminium ion intermediate with the return of the more stable conformation of the enzyme, and **5** is the covalent TS=FdUMP= $\text{CH}_2\text{-H}_4\text{folate}$  complex.

factors. First, the basicity of N-10 of  $\text{CH}_2\text{-H}_4\text{folate}$  could be modified (by rotation, stress, or electronic perturbation of the PABA group) in a manner that could enhance protonation of N-10 and/or the reactivity of the N-10-C-11 bond. Second, the increased basicity of N-10 could enhance formation of a hydrogen bond to the putative general-acid catalyst, A-H. Finally, reactivity could be increased by strain (lengthening) of the N-10-C-11 bond as the cofactor accommodates the transition-state-like conformation of the enzyme. As the 5-iminium ion intermediate **4** is formed, several changes occur: (i) The enzyme adopts a conformation more like the lower energy state of the ground state. (ii) The hydrogen bond between TS and N-10 is broken because of the dramatically lower  $pK_a$  (ca. 4 pH units) of N-10 when a positive charge develops at N-5 (Kallen & Jencks, 1966a). (iii) The general catalyst of the enzyme is left as its conjugate base, A:<sup>7</sup> Finally, covalent bond changes occur (Figure 2) to give the covalent complex **5** which has the PABA ring perturbed by the enzyme and has the hydrogen bond acceptor A: adjacent to N-10 as proposed from Raman resonance studies (Fitzhugh et al., 1986). It is of interest to note that if the normal enzymic reaction is pursued along the remainder of the path in the analogous conversion of dUMP to dTMP, there is a use for the general base (viz., removal of the 5-H of dUMP) that completes the proton inventory.

**Perspectives.** With a complete kinetic and thermodynamic description of all the steps in the reaction of FdUMP and  $\text{CH}_2\text{-H}_4\text{folate}$  with TS and the recently reported three-di-

mensional structure of the enzyme (Hardy et al., 1987), we are now able to approach questions as to how each of these steps is catalyzed. In particular, we are looking forward to studies of how mutagenesis of specific amino acid residues of TS will affect individual steps of the reaction. Our proposal that enzyme-altered environment of the PABA group effects reactivity of  $\text{CH}_2\text{-H}_4\text{folate}$  can also be tested with suitable chemically modified cofactor analogues and by in-depth studies of the PABA perturbation. Finally, inquiries into the three-dimensional structure of the TS=FdUMP= $\text{CH}_2\text{-H}_4\text{folate}$  and/or related complexes may reveal whether our hypotheses on the binding of  $\text{CH}_2\text{-H}_4\text{folate}$  and on the mechanism of 5-iminium ion formation are correct. Although speculative, all of these proposals are amenable to experimental test.

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<sup>7</sup> The apparent enigma of a general acid both serving to protonate the poorly basic N-10 of the cofactor and existing as its conjugate base in neutral media is explained by the proposal that basicity of N-10 is increased in the noncovalent complex.

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## ATP Synthase from Bovine Mitochondria: Sequences of Imported Precursors of Oligomycin Sensitivity Conferral Protein, Factor 6, and Adenosinetriphosphatase Inhibitor Protein<sup>†</sup>

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**ABSTRACT:** Oligomycin sensitivity conferral protein (OSCP), factor 6 (F6), and ATPase inhibitor protein are all components of the ATP synthase complex of bovine mitochondria. They are encoded in nuclear DNA. Complementary DNA clones encoding the precursors of these proteins have been isolated from a bovine library by using mixtures of synthetic oligonucleotides as hybridization probes, and their DNA sequences have been determined. The deduced protein sequences show that the OSCP, F6, and inhibitor proteins have N-terminal presequences of 23, 32, and 25 amino acids, respectively. These presequences are not present in the mature proteins. It is assumed that they serve to direct the proteins into the mitochondrial matrix. The cDNA clones have also been employed as hybridization probes to investigate the genetic complexity of the three proteins in cows and humans. These experiments indicate that the bovine and human inhibitor and bovine F6 proteins are encoded by single genes but suggest the possibility of the presence in both species of more than one gene (or pseudogenes) for the OSCP.

**O**ligomycin sensitivity conferral protein (OSCP), factor 6 (F6), and ATPase inhibitor protein are all associated with the ATP synthase ( $F_1F_0$  ATPase) of bovine mitochondria. This multisubunit enzyme is a complex of about 13 different polypeptides (Stiggal et al., 1978; Lutter et al., 1987; Walker

et al., 1987b) that is anchored in the inner mitochondrial membrane by an intrinsic membrane sector  $F_0$ . The rest of the enzyme, the  $F_1$  complex, which contains the catalytic sites of the enzyme [for reviews see Senior (1978) and Cross (1981)], is in the mitochondrial matrix and is apparently attached to  $F_0$  by a stalk (Senior, 1971; Knowles et al., 1972) composed of, at least in part, the OSCP (MacLennan & Tzagoloff, 1968) and F6 (Fessenden-Radan, 1972). The inhibitor, a small basic protein, binds to  $F_1$  and may well have a physiological role in modulating its activity (Pullman & Monroy, 1963; Asami et al., 1970; Ernster et al., 1973; van de Stadt & van Dam, 1974). Two of the subunits of ATP synthase, ATPase-6 and A6L, both intrinsic membrane proteins, are products of the mitochondrial genome (Fearnley &

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