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Binding of Pyrimidin-2-one Ribonucleoside by Cytidine Deaminase as the Transition-State Analogue 3,4-Dihydrouridine and the Contribution of the 4-Hydroxyl Group to Its Binding Affinity†

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ABSTRACT: Cytidine deaminase, purified to homogeneity from constitutive mutants of *Escherichia coli*, was found to bind the competitive inhibitors pyrimidin-2-one ribonucleoside (apparent $K_i = 3.6 \times 10^{-7}$ M) and 5-fluoropyrimidin-2-one ribonucleoside (apparent $K_i = 3.5 \times 10^{-8}$ M). Enzyme binding resulted in a change of the λ_{max} of pyrimidin-2-one ribonucleoside from 303 nm for the free species to 239 nm for the bound species. The value for the bound species was identical with that of an oxygen adduct formed by combination of hydroxide ion with 1,3-dimethyl-2-oxopyrimidinium (239 nm), but lower than that of a sulfur adduct formed by combination of the thiolate anion of *N*-acetylcysteamine with 1,3-dimethyl-2-oxopyrimidinium (259 nm). The results suggest that pyrimidin-2-one ribonucleoside is bound by cytidine deaminase as an oxygen adduct, probably the covalent hydrate 3,4-dihydrouridine, rather than intact or as an adduct involving a thiol group of the enzyme. In dilute solution at 25 °C, the equilibrium constant for formation of a single diastereomer of 3,4-dihydrouridine from pyrimidin-2-one ribonucleoside was estimated as approximately 4.7×10^{-6}, from equilibria of dissociation of water, protonation of 1-methylpyrimidin-2-one, and combination of the 1,3-dimethylpyrimidinium cation with the hydroxide ion. On the basis of this equilibrium constant and the apparent K_i value observed for pyrimidin-2-one ribonucleoside, the equilibrium constant for dissociation of a single inhibitory isomer of 3,4-dihydrouridine from the enzyme was estimated as 1.2×10^{-12} M, more than 8 orders of magnitude lower than the K_m value of the substrate cytidine. Replacement of the 4-hydroxyl group of 3,4-dihydrouridine, in 3,4-dihydropyrimidin-2-one ribonucleoside (apparent $K_i = 3.0 \times 10^{-5}$ M), reduced its negative free energy of binding by 10.1 kcal/mol.

Cytidine deaminase catalyzes a thermodynamically favorable reaction whose product, uridine, can undergo phosphorolysis to yield pentose derivatives. Thus, the presence of this enzyme in bacteria allows cytidine to serve as a sole carbon source (Hammer-Jespersson & Nygaard, 1976). The bacterial enzyme enhances the rate of hydrolytic deamination of cytidine by a factor of approximately 4×10^{11} (Frick et al., 1987). The activity of this enzyme is not affected by the presence of EDTA but is inhibited reversibly by mersalyl ($K_i = 3 \times 10^{-5}$ M) (Wolfenden et al., 1967) and irreversibly by 5-mercuriocytidine (Ashley & Bartlett, 1984a), suggesting the presence of an essential sulfhydryl group.

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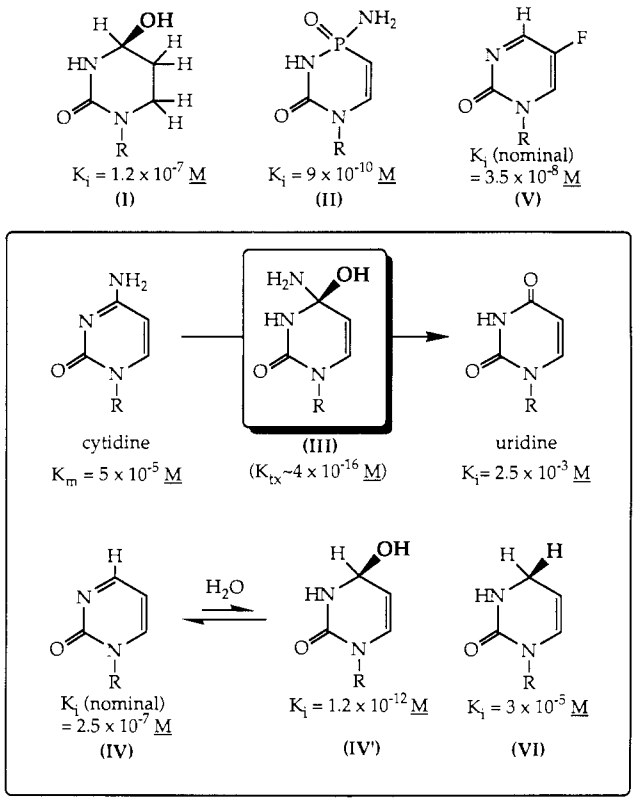

FIGURE 1: One of two possible mechanisms of action of cytidine deaminase, involving direct water attack on cytidine to generate tetrahedral intermediate III. An alternative mechanism would involve double displacement by a thiolate residue at the active site. Inhibitors shown are 3,4,5,6-tetrahydrouridine (I), a phosphapyrimidine ribonucleoside (II), pyrimidin-2-one ribonucleoside (IV), 3,4-dihydrouridine (IV'), 5-fluoropyrimidin-2-one ribonucleoside (V), and 4-hydroxy-3,4-dihydropyrimidin-2-one ribonucleoside (VI). K_{tx} represents the maximal dissociation constant of the altered substrate in the transition state for the catalyzed reaction, estimated as described by Frick et al., (1987). For K_i values, see text. The stereochemical configuration at C-4 is drawn by analogy with the observed stereochemistry at C-6 of adenosine deaminase inhibitor (Kati & Wolfenden, 1989b), but remains to be established for cytidine deaminase.

Considering the ability of model nucleophiles to catalyze deamination of cytidine by adding to the 5,6-double bond (Shapiro & Klein, 1966, 1967; Notari, 1967; Wechter, 1970; Wechter & Kelly, 1970; Shapiro, 1980), it seemed possible that the action of cytidine deaminase might involve addition of water or an enzyme nucleophile at the 5,6-double bond. That mechanism became less attractive when the enzyme was found to catalyze the hydrolytic deamination of 5,6-dihydrocytidine, in which 5,6-addition of a nucleophile could not occur (Evans et al., 1975). A simpler mechanism of action was suggested by the enzyme's observed sensitivity to the competitive inhibitors shown in Figure 1. Thus, 3,4,5,6-tetrahydrouridine (I) was bound by bacterial cytidine deaminase with a K_i value (2.4×10^{-7} M) several orders of magnitude lower than K_i values observed for uridine ($K_i = 2.5 \times 10^{-3}$ M) or 5,6-dihydrouridine ($K_i = 3.4 \times 10^{-3}$ M) (Cohen & Wolfenden, 1971), and an even higher affinity ($K_i = 9 \times 10^{-10}$ M) was observed for a phosphapyrimidine ribonucleoside (II) (Ashley & Bartlett, 1984b). With a tetrasubstituted atom at the 4-position, compounds I and II share some structural resemblance to a potential reaction intermediate (III) formed by 3,4-addition of water to cytidine that might approach the transition state in cytidine deamination by simple water attack (Figure 1).

This paper describes an effort to characterize the interaction of bacterial cytidine deaminase with pyrimidin-2-one ribonucleoside (IV) and 5-fluoropyrimidin-2-one ribonucleoside (V). These compounds, previously shown to act as competitive inhibitors of yeast and mammalian cytidine deaminases, exhibited K_i values somewhat lower than the K_m value of cytidine (McCormack et al., 1980). With a hydrogen atom at C-4, these compounds are less demanding in their space-filling requirements than the substrate or product and could serve as simple substrate analogues. A second possibility is that these compounds might undergo reversible addition at the 3,4- (and possibly the 5,6-) double bond by an enzyme nucleophile such as the essential sulfhydryl group whose presence had been suggested by the susceptibility of the enzyme to inhibition by mercurials. Fluorine substitution would be expected to promote sulfhydryl addition, offering a possible explanation of the somewhat superior binding affinity of V as compared with IV, as described by McCormack et al. (1980). A third possibility is that these inhibitors might be bound as their 3,4-covalent hydrates such as IV'. Fluorine substitution would also be expected to promote hydration of V, affording an alternative explanation of its superiority in binding affinity to IV. The interest of this third possibility was heightened by the likelihood that equilibria of formation of covalent hydrates such as IV' are extremely unfavorable in free solution, so that if these were the forms actually bound, they would need to possess extremely high affinities to account for the inhibition observed. It was therefore of interest to determine the approximate positions of model equilibria in free solution.

A decision between alternative mechanisms of binding of these inhibitors, whether intact or as an oxygen or sulfur adduct, could indicate the mechanism that is likely to be followed during enzymatic deamination of cytidine. We sought to make this distinction by examining UV absorption spectra of these inhibitors, complexed with highly purified enzyme from mutant bacteria that overproduce cytidine deaminase (Munch-Petersen et al., 1973). As models for alternative structures of the bound inhibitor and to estimate the equilibrium constant for formation of IV' in free solution, we also investigated the formation of adducts formed by combination of the model compound 1,3-dimethyl-2-oxopyrimidinium cation with hydroxide ion and with the anion of *N*-acetylcysteamine, as shown in Figure 2.

Materials and Methods

The inhibitors pyrimidin-2-one ribonucleoside (IV), 5-fluoropyrimidin-2-one ribonucleoside (V), 3,4-dihydropyrimidin-2-one ribonucleoside (VI), and 3,4-dihydro-4-(hydroxymethyl)pyrimidin-2-one ribonucleoside were prepared as described by Liu et al. (1981), McCormack et al. (1980), and Kim et al. (1986). 1-Methylpyrimidin-2-one and 1,3-dimethyl-2-oxopyrimidinium chloride were prepared by the method of Tee and Endo (1974).

The activity of cytidine deaminase was monitored by following the decrease in UV absorbance when cytidine was converted to uridine, at 280 nm ($\Delta\epsilon = -3600$), in potassium phosphate buffer (0.025 M, pH 6.8) at 25 °C. Inhibition constants were determined from double-reciprocal plots of enzyme activity as a function of changing substrate concentration in the presence and absence of inhibitors. Ultraviolet difference spectra of enzyme–inhibitor complexes were determined under similar conditions, by comparison of the absorption spectra of enzyme and inhibitor, mixed and unmixed, by use of cuvettes with two compartments and a Coleman Model 124 double-beam spectrophotometer.

Cytidine deaminase was prepared from strain CGSc 5903 of *Escherichia coli* [equivalent to strain Sϕ-744 of Munch-Petersen et al. (1973)], obtained from the *E. coli* Stock Center

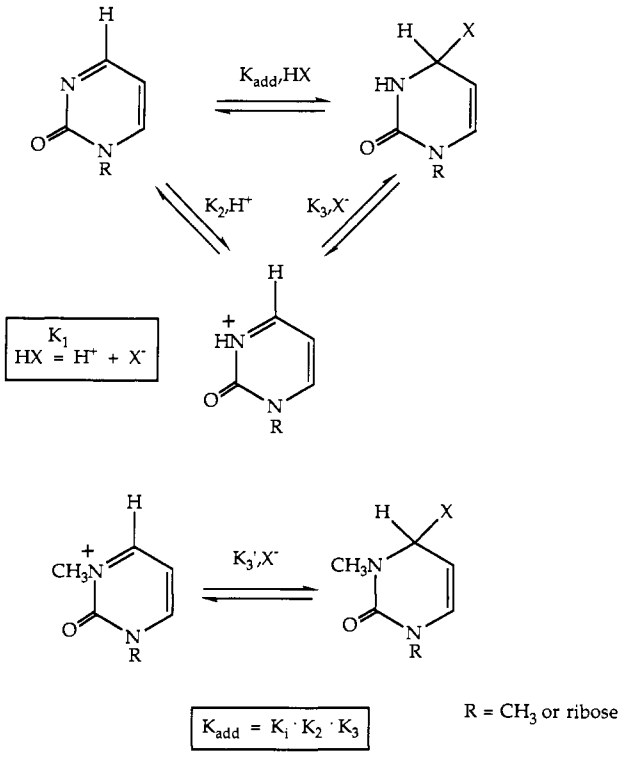

FIGURE 2: Equilibrium of addition of a nucleophile HX (water or a mercaptan in the present experiments), analyzed in terms of successive equilibrium constants for dissociation of HX to form a proton and a nucleophilic anion (K_1), addition of a proton to the C=N bond (K_2), and addition of the nucleophilic anion X^- (hydroxide or thiolate in the present experiments) to the protonated C=N bond (K_3). The third of these equilibria can be modeled by addition of that anion to the quaternary amine formed by N-methylation (K_3').

of Yale University. Cells were grown overnight in minimal medium containing uridine (1×10^{-2} M), L-leucine, L-valine, and L-methionine (0.3 mg/mL each), thiamin (10 μg/mL), and streptomycin (25 μg/mL at 37 °C with vigorous shaking. The resulting suspension (25 mL) was used to inoculate 750 mL of LB broth (Gibco, Inc.) containing streptomycin (25 μg/mL). After vigorous shaking for 16 h at 37 °C in 2-L flasks, cells were collected by centrifugation (7000*g* × 30 min at 4 °C) and suspended in two 2-L flasks, each containing 750 mL of the original minimal medium, in which they were then incubated for 20 h. After harvesting by centrifugation, the cells were digested with lysozyme (0.5 mg/mL) for 1 h as described by Ashley and Bartlett (1984a) and then lysed by being heated to 60 °C for 1 min. This step and subsequent steps were carried out in the presence of 2-mercaptoethanol (10^{-2} M). After centrifugation (20000*g* × 45 min at 4 °C), the supernatant solution was adjusted to 80% saturation with ammonium sulfate. After centrifugation, the precipitate was successively extracted with solutions (2 mL/g of cells) of ammonium sulfate at 55%, 45%, and 40% of saturation in potassium phoshate buffer (0.025 M, pH 6.8), containing 10^{-3} M EDTA. The last of these extracts was adjusted to 55% saturation with ammonium sulfate, and the precipitate was collected by centrifugation and suspended in and dialyzed against potassium phosphate buffer (0.1 M, pH 6.8). After any precipitate had been removed by centrifugation and discarded, this material was applied to a column (1 × 50 cm) of AcA-34 Ultrogel (LKB-Pharmacia, Inc.) and eluted with the same buffer at a flow rate of 10 mL/h. Active fractions were diluted by addition of 3 volumes deionized water and applied to a column (1 × 17 cm) of DEAE-Sephacel (LKB-Pharmacia, Inc.) that had been equilibrated with potassium phosphate buffer (0.025 M, pH 6.8). Activity was eluted with a linear gradient of the same buffer, increasing to a concentration of 0.25 M over a volume of 500 mL. Active fractions were dialyzed against piperazine hydrochloride buffer (2.5×10^{-2} M, pH 5.5) and applied to a chromatofocusing column (1 × 50 cm) containing PBE-74 gel (LKB-Pharmacia, Inc.) that had been previously equilibrated with piperazine buffer and then washed with Polybuffer 74 hydrochloride (80 mL, diluted 1:9 with water; pH 3.8). Activity was eluted with this last solution and collected in tubes that contained potassium phosphate buffer (0.15 mL 1 M, pH 8.0) to neutralize the pH. The purity of this final preparation was established by SDS–PAGE (Laemmli, 1970), staining being with silver. Molecular weight standards were obtained from Sigma Chemical Co. p*I* values were determined on a pH 3–9 plate with a pHastgel electrophoresis apparatus (Pharmacia-LKB, Inc.) operating at 500 V·h.

Table I: Purification of Cytidine Deaminase

step	vol (mL)	units/mL	mg/mL	units/mg	recovery (%)
crude	108	34	1.84	18.5	(100)
$(NH_4)_2SO_4$	3	751	40	19	52
AcA-34	7	226	7.5	30	36
DEAE	20	50	0.36	139	23
PBE-74	40	22.5	0.095	237	21

Table II: Changes in Specific Activity of Cytidine Deaminase after Media Transfer

time (h)	A_{260}	A_{280}	A_{595}	units/mL
2	66	36	2.5	4.3
4	84	45	3.5	10
10	72	42	3.6	16
24	75	42	4.4	18.5

RESULTS

Purification of Cytidine Deaminase. The purification procedure, described under Materials and Methods, is summarized in Table I. Approximately 330 units of crude enzyme was obtained from each gram of bacteria, representing a substantial increase over the yield from induced bacteria reported by Vita et al. (1985). After transfer from LB broth to minimal medium, these cells did not undergo further growth, as indicated by stable turbidity readings (Table II), but their content of cytidine deaminase continued to increase for some time after transfer. Cells grown in minimal medium and transferred to fresh minimal medium contained less enzyme (7.4 units/mL) per unit volume of medium than did cells transferred from LB broth to minimal medium (18.5 units/mL). Enzyme from cells cultured only in minimal medium showed lower specific activity than enzyme from cells cultured first in LB broth. The specific activity of the final preparation (220 units/mL) was somewhat higher than specific activities of material purified by earlier affinity (Ashley & Bartlett, 1984a) and electrophoretic (Vita et al., 1985) methods.

This material, in which faint bands of contaminating protein could still be observed on SDS–PAGE gels stained with silver, with loadings in excess of 100 ng of protein (Figure 3), has recently yielded single crystals of high quality (Betts et al., 1989). The molecular weight of the pure enzyme, estimated by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970), was 31 400, slightly lower than values of 35 000 reported by Ashley and Bartlett (1984a) and 33 000 reported by Vita et al. (1985). The observed p*I* value was 4.38, in close agreement with the value of 4.35 reported by Vita et al. (1985).

Enzyme Binding and UV Absorption Spectra of Bound Pyrimidin-2-one Ribonucleoside and 5-Fluoropyrimidin-2-one Ribonucleoside. Pyrimidin-2-one ribonucleoside and 5-

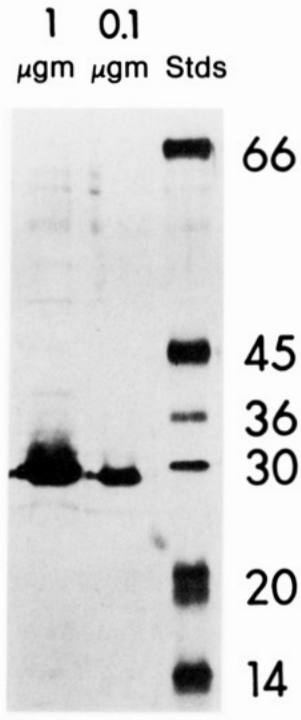

FIGURE 3: 10% SDS–polyacrylamide gel electrophoresis of purified cytidine deaminase, according to the procedure of Laemmli (1970), silver-stained by the method of Ray et al. (1981).

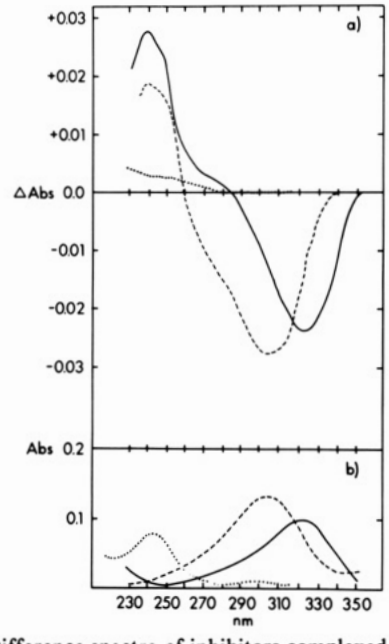

FIGURE 4: Difference spectra of inhibitors complexed with cytidine deaminase: (a) The inhibitor (10^{-5} M) and an equal concentration of cytidine deaminase were placed in separate compartments of a tandem cuvette, and the base-line was determined. The contents were then mixed, and the change in spectrum was recorded. (b) Spectra of inhibitors alone, at a concentration of 2×10^{-6} M. Inhibitors were pyrimidin-2-one ribonucleoside (– –), 5-fluoropyrimidin-2-one ribonucleoside (—), and 4-(hydroxymethyl)-3,4-dihydropyrimidin-2-one ribonucleoside (•••).

fluoropyrimidin-2-one ribonucleoside served as linear competitive inhibitors of bacterial cytidine deaminase. K_i values, observed at pH 6.8 and 25 °C, were 3.6×10^{-7} M for pyrimidin-2-one ribonucleoside and 3.5×10^{-8} M for 5-fluoropyrimidin-2-one ribonucleoside.

Reversible changes in UV absorption spectra were observed when these inhibitors were mixed with cytidine deaminase, the unmixed components being used as a reference. Figure 4 shows difference spectra obtained when inhibitors, present at an initial concentration of 1×10^{-5} M, were mixed with an equal volume of cytidine deaminase at the same concentration (1×10^{-5} M, 0.31 mg/mL) in 0.1 M potassium phosphate buffer, pH 6.8, at 25 °C), giving a final concentration of 5×10^{-6} M for both components of the mixture. Absorption spectra of the inhibitors alone, at a concentration of 2×10^{-5} M, are shown at the bottom of Figure 4. The wavelength of maximum absorption of pyrimidin-2-one ribonucleoside (broken line) changed from 303 to 239 nm upon binding by the enzyme. The wavelength of maximum absorption of 5-fluoropyrimidin-2-one ribonucleoside (solid line) changed from 323 to 240 nm upon binding by the enzyme. In a control experiment involving 3,4-dihydro-4-(hydroxymethyl)pyrimidin-2-one ribonucleoside (dotted line), a strong inhibitor that cannot undergo addition reactions at the 3,4-position (Kim et al., 1986), no change in the wavelength of maximum absorption was observed, although there was a slight increase in extinction coefficient.

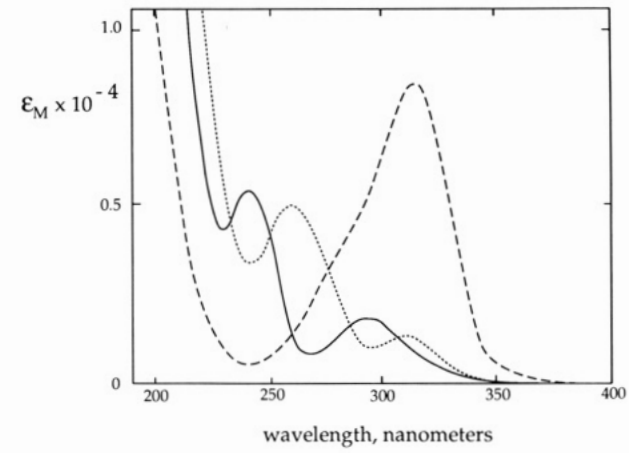

FIGURE 5: UV absorption spectra of 1,3-dimethylpyrimidinium chloride: broken line (– –) = cation, at pH 4.0; dotted line (•••) = thiolate adduct formed in the presence of 0.05 M *N*-acetylcysteine in potassium phosphate buffer (0.5 M, pH 6.8); solid line (—) = pseudobase, in 0.1 M KOH.

UV Absorption Spectra of Model Adducts. Earlier work (Tee & Paventi, 1981) has established that equilibria of addition of water to derivatives of pyrimidin-2-one are unfavorable, so that pyrimidin-2-one ribonucleoside would not be expected to undergo addition of oxygen or sulfur nucleophiles to a significant extent in neutral aqueous solution. However, compounds with similar properties can be prepared, at least in principle, by reaction of nucleophilic anions with these compounds, after "fixing" their hydrogen atoms by N-methylation, to form a quaternary ammonium cation. We therefore examined the products of addition of hydroxide ion and the anion of *N*-acetylcysteamine to the chloride salt of 1,3-dimethyl-2-oxopyrimidinium, whose equilibria are described below. When the 1,3-dimethyl-2-oxopyrimidinium cation was titrated with hydroxide ion, the wavelength of maximum absorption of the product was 239 nm, in good agreement with the value reported earlier by Tee and Endo (1976). When the 1,3-dimethyl-2-oxopyrimidinium cation was titrated with the anion of *N*-acetylcysteamine in the presence of 0.1 M phosphate buffer at pH 6.8, the wavelength of maximum absorption of the product was 259 nm. Figure 5 shows the changes in the spectrum that were observed. UV absorption maxima of these model oxygen and sulfur adducts, with those of the enzyme's complex with the inhibitor 2-oxopyrimidine ribonucleoside (IV), are compared in Table III. The results suggested that IV was bound by cytidine deaminase

Table III: λ_{max} Values (nm) of Models for Enzyme-Bound Pyrimidin-2-one Ribonucleoside (IV), at 25 °C and Ionic Strength 0.10

models	
1-methylpyrimidin-2-one	
neutral species	**302** (model for neutral IV in free solution)
cation	312
1,3-dimethyl-2-oxopyrimidinium	
cation	316
OH^- adduct (K_d = 1.4 × 10^{-7} M)[a]	**239** (model for an oxygen adduct of IV)
N-Ac-Cys-S^- adduct (K_d = 3 × 10^{-6} M)[b]	259 (model for a sulfur adduct of IV)
inhibitor	
pyrimidin-2-one ribonucleoside (IV)	
neutral species in free solution	**303**
enzyme bound	**239**

[a] Calculated from the apparent pK_a value, 7.15, at which pseudobase formation occurs. [b] Measured at pH 6.8 and expressed in terms of the concentration of thiol (pK_a = 9.9) that was present in anionic form and of 1,3-dimethyl-2-oxopyrimidinium that was present in cationic form at that pH value.

as an oxygen adduct, perhaps the covalent hydrate IV′.

Estimated Equilibrium Constant for Hydration of 1-Methylpyrimidin-2-one. Even though IV′ is expected to be present at very low concentrations in solution, the equilibrium constant for its formation can be estimated by an indirect method that appears to have been recognized for the first time by Bunting (1966) [see also Jones and Wolfenden (1986)]. According to this method, illustrated for the present case in Figure 2, the covalent hydration of a C=N bond is considered to proceed in three stages: (1) ionization of water, (2) addition of a proton at nitrogen, and (3) combination of the protonated species with hydroxide ion. The numerical product of the equilibrium constants for these three steps should be equivalent to the equilibrium constant for hydration. The first two equilibrium constants (K_1 and K_2) can be obtained directly from the pK_a values of H_2O and the conjugate acid of the N-protonated species. The third equilibrium constant is not directly accessible, because the N-protonated species is present only in vanishingly small amounts at pH values where the concentration of the hydroxide ion becomes significant. Its value can, however, be estimated indirectly by measuring the affinity of the parent compound, quaternized by methylation, for hydroxide ion, i.e., the equilibrium constant for pseudobase formation. This method involves the assumption that a quaternized ring $=NCH_3^+-$ group and a ring $=NH^+-$ group are approximately equivalent in their electronic effects. That assumption is supported by the similar hydroxide ion affinity of the conjugate acid of the 3-methylquinazolinium cation and the 3-protonated conjugate acid of quinazoline itself (Albert et al., 1961).

Applying this procedure to 1-methylpyrimidin-2-one, we obtained a pK_a value of 2.42 for the conjugate acid of 1-methylpyridin-2-one, corresponding to a value of 263 M^{-1} for K_2, at 25 °C and ionic strength 0.10. The affinity of the 1,3-dimethyl-2-oxopyrimidinium cation for the hydroxide ion at 25 °C was obtained by spectrophotometric titration of its chloride salt under the same conditions. Its apparent pK_a value was 7.15, in good agreement with a value of 7.11 at 24 °C reported earlier by Tee and Endo (1976). This value corresponds to a formation constant (K_3, Figure 2) of 7.1 × 10^6 M^{-1} for the pseudobase from the 1,3-dimethyl-2-oxopyrimidinium cation and the hydroxide ion. Multiplying K_1, the dissociation constant of water (10^{-14} M^2), by these values for K_2 (263 M^{-1}) and K_3 (7.1 × 10^6 M^{-1}), the equilibrium constant for hydration of 1-methyl-2-oxopyrimidine at 25 °C and ionic strength 0.10 was estimated as 1.86 × 10^{-5}, expressed in terms of unit water activity.

In order to estimate the equilibrium constant for reaction of pyrimidin-2-one ribonucleoside (IV) with solvent water, it seemed appropriate to make correction for the fact that the 1,3-dimethyl-2-oxopyrimidinium cation (unlike the conjugate acid of IV) has two equivalent carbon atoms (4 and 6) at which uptake of a hydroxide ion can occur. This presumably doubles the likelihood of its association with the hydroxide ion. For hydroxide association with only the 4-position (the situation in the ribonucleoside IV), the equilibrium constant would then be only half as great as the value observed for the 1,3-dimethyl-2-oxopyrimidinium cation. A second correction seems appropriate to allow for the fact that the hydroxide ion can add at the 4-position to either of the two faces of the pyrimidine ring. However, because of the high affinity of IV′ for the enzyme's active site, it seems reasonable to suppose that only one of its two diastereomers is likely to be bound by the enzyme. We wish to estimate the probability of forming only the one diastereomer of IV′ that is presumably active, and this is presumably only half as great as the probability of forming both diastereomers, which are probably present at similar abundance in solution. Making these two corrections, each amounting to a factor of 2, we estimate that the equilibrium constant for hydration of IV in dilute solution, to form the single isomer of IV′ that we presume to be active, may be approximately 4.7 × 10^{-6} (or 8.4 × 10^{-8} M^{-1} on the basis of 55.5 M water).

Inhibition by 3,4-Dihydropyrimidin-2-one Ribonucleoside. Structural features distinguishing IV′ from IV that might help to explain the apparently very high affinity of IV′ include (a) the proton at N-2, which could form a hydrogen bond to the enzyme; (b) distortion of the ring from its original planar configuration, possibly altering various noncovalent contacts; and (c) the hydroxyl group at C-4, which could form hydrogen bonds to the enzyme. If ring distortion or the proton at N-2 were important for tight binding, then the enzyme would also be expected to interact strongly with 3,4-dihydropyrimidin-2-one ribonucleoside, lacking the 4-hydroxyl group. Differences in binding affinity between 3,4-dihydropyrimidin-2-one ribonucleoside and IV′ should be largely attributable to the presence of the 4-hydroxyl group in IV′. To test these alternatives, we examined the inhibition of cytidine deaminase by 3,4-dihydropyrimidin-2-one ribonucleoside. This compound, previously shown to inhibit yeast and mammalian enzymes (Kim et al., 1986), proved to be a simple competitive inhibitor of bacterial cytidine deaminase, with a K_i value of 3.0 × 10^{-5} M.

Discussion

Probable Binding of Pyrimidin-2-one Ribonucleoside as 3,4-Dihydrouridine. The λ_{max} of enzyme-bound pyrimidin-2-one ribonucleoside, indicated by the difference spectrum of the enzyme–inhibitor complex, was 240 nm. This value was similar to the value (239 nm) observed for the oxygen adduct formed by combination of hydroxide ion with 1,3-dimethyl-2-oxopyrimidinium but much lower than the value (259 nm) observed for the sulfur adduct. These results strongly suggest that IV is bound as its covalent hydrate, 3,4-dihydrouridine (IV′), or as an adduct formed by some other oxygen nucleophile at the enzyme's active site, and not as a sulfur adduct. In considering whether IV is bound as the covalent hydrate 3,4-dihydrouridine (IV′) or as an adduct formed by some other oxygen nucleophile present at the enzyme's active site, one may note the resemblance between IV′ and the strong inhibitors I and II, in which the tetrahedral arrangement of substituents

at C-4 resembles that of IV'.[1]

Taken together, these results suggest that IV is bound as the covalent hydrate 3,4-dihydrouridine (IV'). It may also be worth noting that binding of IV does not appear to involve enzyme addition at *both* the 3,4- and the 5,6-double bonds. If that had been the case, then the spectrum of the bound inhibitor would have been expected to have resembled that of 3,4,5,6-tetrahydrouridine, which is devoid of absorption maxima above 220 nm (Wentworth and Wolfenden, unpublished observations). The conclusion that compounds IV and V are probably bound as their covalent 3,4-hydrates IV' and V' must be considered tentative and remains to be tested by crystallographic studies now in progress (Betts et al., 1989). If correct, this conclusion would imply that cytidine undergoes enzymatic deamination by direct water attack.

Binding Affinity of 3,4-Dihydrouridine (IV') and Effective Concentration of Water at the Active Site. If compound IV' resembles a hydrate intermediate (III) that approaches the transition state for cytidine deamination, it is of interest to consider the possible magnitude of the affinity of this species for the active site of cytidine deaminase. The equilibrium constant for hydration of IV in dilute solution, to form the single isomer of IV' that may be presumed to be active, is approximately 4.7×10^{-6} on the basis of unit water activity (or 8.4×10^{-8} M^{-1} on the basis of 55.5 M water). From this equilibrium constant and the apparent K_i value of IV, 2.5×10^{-7} M, the true K_i value of the inhibitory diastereomer of IV' was estimated as 1.2×10^{-12} M roughly 8 orders of magnitude lower than the K_m value of cytidine [2.4×10^{-4} M (Cohen & Wolfenden, 1971)]. As the enzyme enhances the reaction rate by a factor of approximately 4×10^{11} (Frick et al., 1987), compound IV' appears to capture much of the negative free energy of binding expected of an ideal transition-state analogue for this reaction.

From the change in UV absorption spectrum observed, it seems probable that the majority of IV is converted to IV' at the enzyme's active site, where the effective equilibrium constant for hydration appears, conservatively, to be greater than unity. Since the equilibrium constant for conversion of IV to IV' (active isomer only) in dilute solution is approximately 8×10^{-8} M^{-1} on the basis of the actual molarity of water, the effective concentration of water at the active site can be estimated as at least 10^7 M, with respect to the hydration of IV. The present results do not completely exclude, although they seem to render unlikely (see earlier discussion), the possibility that some other nucleophile, such as an active site serine residue, might have added to IV in place of water. If that were the case, then the effective concentration of that nucleophile at the active site would need to be very large.

Apparent Contribution of the 4-Hydroxyl Group to the Binding of 3,4,5,6-Tetrahydrouridine by Cytidine Deaminase. The K_i value of 3,4-dihydropyrimidinone ribonucleoside is 3×10^{-5} M, higher than the estimated K_i value of IV' ($K_i = 1.2 \times 10^{-12}$ M) by a factor of 2.5×10^7 (equivalent to 10.1 kcal/mol in free energy). As these compounds differ only in replacement of the 4-hydroxyl group by hydrogen, the difference between their binding affinities provides some indication of the contribution made by that hydroxyl group to the binding affinity of IV' in aqueous solution.

This difference in apparent binding affinities becomes more pronounced if the effects of solvation are considered, since hydroxylic compound IV' is substantially more difficult to remove from water than is the hydrogen compound. Because IV' is so tightly bound, it seems reasonable to suppose that it fits the active site snugly, with removal of solvent water from the points at which this ligand makes contact with the active site. The cost of removing solvent water from the active site is probably roughly the same for either ligand, but the hydroxylic compound is more hydrophilic than the hydrogen compound by a factor of approximately 10^5 (Butler, 1937). Accordingly, the *desolvated* ligands would be expected to differ in binding affinities by a factor of approximately $(10^5)(2.5 \times 10^7) = 2.5 \times 10^{12}$, or 17 kcal/mol in free energy. Enthalpies of hydrogen-bond formation in the vapor phase suggest that at least one charged group at the active site is needed to produce this level of binding discrimination (Kati & Wolfenden, 1989).

Relative Binding Affinities of Uridine, 3,4-Dihydrouridine, and 3,4,5,6-Tetrahydrouridine. 3,4-Dihydrouridine (apparent $K_i = 1.2 \times 10^{-12}$ M) appears to be bound almost (2×10^8)-fold more tightly than the substrate cytidine ($K_m = 2.1 \times 10^{-4}$ M). As bacterial cytidine deaminase produces an apparent rate enhancement of approximately (4×10^{11})-fold (Frick et al., 1987), this inhibitor appears to capture roughly 70% of the minimal extra negative free energy of binding expected of an ideal transition-state analogue , as compared with that of the substrate. However, if this hypothesis is to be regarded as acceptable, then the very much weaker binding observed for 3,4,5,6-tetrahydrouridine ($K_i = 1.2 \times 10^{-7}$ M) (Figure 1) requires explanation. Bacterial cytidine deaminase has been found to act on 5,6-dihydrocytidne at a limiting rate equivalent to 15% of its rate of action on the normal substrate cytidine (Evans et al., 1975). The K_m value of 5,6-dihydrocytidine was 1.1×10^{-4} M only a little higher than that of cytidine ($K_m = 4.0 \times 10^{-5}$ M) (Evans et al., 1975). Why then should 3,4,5,6-tetrahydrouridine ($K_i = 1.2 \times 10^{-7}$ M), which stands in relation to the substrate 5,6-dihydrocytidine as 3,4-dihydrouridine stands in relation to the substrate cytidine, not be bound almost as tightly as 3,4-dihydrouridine ($K_i = 1.2 \times 10^{-12}$ M)?

An answer to this apparent paradox may lie in the relative rates of the *nonenzymatic* reactions. In neutral solution at 25 °C, the nonenzymatic deamination of 5,6-dihydrocytidine proceeds with a rate constant of 1.1×10^{-4} s^{-1} (Evans et al., 1975), very much greater than the estimated rate of nonenzymatic deamination of cytidine, 3×10^{-10} s^{-1} (Frick et al., 1987). Based on our present estimate of the specific activity of the pure enzyme, the turnover number of 5,6-dihydrocytidine is approximately 15 s^{-1}, corresponding to enhancement of the nonenzymatic rate of deamination of 5,6-dihydrocytidine by a factor of 1.4×10^5.

Because the nonenzymatic deamination of 5,6-dihydrocytidine is so rapid and the enzyme's enhancement of the rate of this reaction so small, the affinity expected of an ideal transition-state analogue for this reaction is very much less than that expected of an ideal transition-state analogue for cytidine deamination. According to this criterion, 3,4,5,6-tetrahydrouridine may be a more effective inhibitor than had been supposed. The minimal factor by which an ideal transition-state analogue should be bound more tightly than the substrate 5,6-dihydrocytidine is 1.4×10^5, whereas the factor by which 3,4,5,6-tetrahydrouridine ($K_i = 1.2 \times 10^{-7}$ M) is

[1] Compounds I and II might conceivably undergo substitution and addition reactions, respectively, when they are bound by cytidine deaminase, in such a way as to lead to covalently bound species with the appropriate hybridization. However, it appears highly unlikely that these reactions would proceed sufficiently rapidly and reversibly to lead to competitive inhibition. In addition, Ashley and Bartlett (1984b) observed no significant change in the ultraviolet spectrum when compound II was bound by bacterial cytidine deaminase, indicating that compound II was bound intact.

bound more tightly than the substrate 5,6-dihydrocytidine (K_m = 1.13 × 10^{-4} M) is 0.94 × 10^3. Expressed in terms of free energy, 3,4,5,6-tetrahydrouridine appears to capture roughly 60% of the minimal extra negative free energy of binding expected of an ideal transition-state analogue for the deamination of 5,6-dihydrocytidine. In this sense, 3,4,5,6-tetrahydrouridine appears to be nearly as effective an inhibitor with respect to the deamination of 5,6-dihydrocytidine as is 3,4-dihydrouridine with respect to the deamination of cytidine.

Comparison with Results Obtained for Cytosine Deaminase. It is of interest to compare the present results with those obtained on an enzyme that catalyzes hydrolytic deamination of the free base, cytosine. In the presence of a partly purified preparation of cytosine deaminase from baker's yeast, Kornblatt and Tee (1986) observed a blue shift in the absorption maximum of the free base, 5-bromo-2-oxopyrimidine. They attributed this change in spectrum to binding of the heterocycle partly in hydrated form. Since spectra of adducts involving nucleophiles other than oxygen were not available for comparison, alternative structures of the bound inhibitor were not considered. In free solution, hydration of 5-bromo-2-oxopyrimidine was slow enough to measure, varying in rate with pH. The enzyme was observed not to catalyze the hydration reaction, but the onset of inhibition was rapid. Because the extent of apparent inhibition by the uncharged inhibitor did not vary appreciably with changing pH, Kornblatt and Tee inferred that inhibition was largely due to the unhydrated species, whose abundance varies only slightly with changing pH in the range they considered. According to their reasoning, if inhibition had been mainly due to the hydrated inhibitor, then the extent of inhibition should have varied acutely with changing pH. They were led to the paradoxical conclusion that the hydrate was not the major inhibitor but that the bound form, presumably occupying the active site, had nevertheless undergone extensive hydration.

These findings may be open to a simpler interpretation. Kornblatt and Tee observed that the rate of onset of inhibition by 2-oxopyrimidine was instantaneous, as in the present experiments on inhibition of cytidine deaminase by 2-pyrimidine ribonucleoside and in earlier experiments on the interaction of adenosine deaminase with nebularine (Kurz & Frieden, 1987; Jones et al., 1989). In the case of adenosine deaminase, nebularine appears to be bound as the hydrated species, yet the concentration of the hydrated species in free solution is several orders of magnitude too low to allow mechanical diffusion of the hydrated inhibitor from solution to be consistent with the observed rapid rate of onset of inhibition. One is led to conclude that the inhibitor is taken up as the unhydrated species and that hydration occurs rapidly within the active site in a process parallel to generation of a hydrated intermediate in the normal catalytic process (Scheme I). The enzyme is never called upon to release the hydrated inhibitor, as would be necessary before true catalysis could be said to have occurred. Indeed, the slow binding of stable inhibitors related in structure to hydrated intermediates suggests that there are severe kinetic barriers to binding and release of compounds of this kind for both cytidine and adenosine deaminases.

Scheme I

$$E + \text{anhyd} \overset{\text{step 1}}{\rightleftharpoons} E\text{–anhyd}$$

$$E\text{–anhyd} + H_2O \overset{\text{step 2}}{\rightleftharpoons} E\text{–hydrate}$$

If the same were also true of cytosine deaminase, this would remove the requirement that the hydrated inhibitor be formed in a pH-dependent process *preceding* enzyme binding. If it is postulated that an enzyme takes up an inhibitor in an unhydrated form (as in cytidine and adenosine deaminases), then the principle of microscopic reversibility requires that the enzyme must also release the inhibitor in its unhydrated form. The observations of Kornblatt and Tee could then be reconciled with uptake of unhydrated 5-bromo-2-oxopyrimidine by cytosine deaminase, and its conversion at the active site to 5-bromo-3,4-dihydrouracil (or 1,6-dihydrouracil), without requiring that cytosine deaminase be capable of overt catalysis of the conversion of free 5-bromo-2-pyrimidinone to the free hydrate that is relatively rare (ca. 4%) in solution. The difference spectrum observed by these authors suggests that the bound form was extensively hydrated.

Binding of 5-bromo-2-pyrimidinone as the hydrate would also help to explain the tighter binding observed by Kornblatt and Tee for 5-bromo-2-pyrimidinone (apparent K_i = 2.6 × 10^{-7} M) than for 2-pyrimidinone itself (apparent K_i = 4.9 × 10^{-6} M), since these authors found that bromination markedly enhances the equilibrium constant for hydration. Tee and Paventi (1980) had shown that the equilibrium constant for hydration of 2-pyrimidinone is 5 × 10^{-4}. Combining this with the K_i values observed for 2-pyrimidinone, the true K_i value of its hydrate (3,4-dihydrouracil) would than be 2.5 × 10^{-9} M, almost 6 orders of magnitude lower than the K_m value of cytosine.

Comparison with Results Obtained for Adenosine Deaminase. Bacterial cytidine deaminase is strikingly similar to mammalian adenosine deaminase, in terms of the rate enhancement that it produces (Frick et al., 1987) and its high affinity for a hydrated analogue of the substrate (Jones et al., 1989). In each case a hydrated analogue generated from an apparently simple competitive inhibitor in a thermodynamically unfavorable hydration reaction that resembles a partial reaction in the normal catalytic process, places a hydrogen atom in the position that would be occupied by the substrate's leaving group. Because the enzyme is nonspecific with respect to the leaving group, this substitution of hydrogen for a hydroxyl substituent is expected to be well-tolerated. In contrast, the hydroxyl group of the hydrated inhibitor appears to play a critical role in binding, contributing by its presence more than 9 kcal to the negative free energy of binding as observed in water. The nature of the attractive forces responsible for this interaction remain to be determined, but it seems reasonable to infer that at least one charged active site residue may be required in order to account for the binding discrimination that is observed [for more extended discussion, see Kati and Wolfenden (1989b)].

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In Vitro Conversion of Formate to Serine: Effect of Tetrahydropteroylpolyglutamates and Serine Hydroxymethyltransferase on the Rate of 10-Formyltetrahydrofolate Synthetase†

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ABSTRACT: Serine hydroxymethyltransferase and C_1-tetrahydrofolate synthase catalyze four reactions which convert formate and glycine to serine. The one-carbon carrier in these reactions if tetrahydropteroylglutamate which is regenerated in the coupled reaction and thus can be used in catalytic concentrations with respect to serine synthesis. The rate of serine synthesis is followed by the oxidation of NADPH during reduction of the intermediate 5,10-methenyltetrahydropteroylglutamate. K_m values for the substrates of cytosolic serine hydroxymethyltransferase and the 10-formyltetrahydrofolate synthetase activity of the trifunctional enzyme C_1-tetrahydrofolate synthase were determined. This included the values for the polyglutamate forms of tetrahydropteroylglutamate containing from one to six glutamate residues. The results suggest that the synthetase active site binds the polyglutamate forms of the coenzyme synergistically with respect to formate and ATP. Using saturating levels of all substrates, the k_{cat} values for the serine hydroxymethyltransferase and 10-formyltetrahydrofolate synthetase activities were also determined. The synthetase reaction is the rate-determining step in the conversion of formate to serine. The effect of glutamate chain length and the concentration of serine hydroxymethyltransferase were studied with respect to the rate of serine formation. Tetrahydropteroylmonoglutamate gave slower than expected rates which is attributed to its inhibition of the reduction of the intermediate 5,10-methenyltetrahydropteroylglutamate. This inhibition was not a factor with the di- through hexaglutamate forms of the coenzyme. The addition of an excess of serine hydroxymethyltransferase was predicted to lower the rate of the formation of serine by lowering the concentration of free coenzyme in the assay. However, activation of the rate was observed which was at least 2-fold greater than the predicted rate. This increase in predicted rate appears to result from an interaction between C_1-tetrahydrofolate synthase and serine hydroxymethyltransferase. The in vivo concentrations of serine hydroxymethyltransferase and C_1-tetrahydrofolate synthase in rabbit liver were determined.

The stereospecific incorporation of formate into the C_3 carbon of L-serine has been demonstrated to occur under physiological conditions (Biellmann & Schuber, 1970). The enzymes involved in this conversion are the multifunctional enzyme C_1-THF synthase[1] and SHMT (EC 2.1.2.1). C_1-THF synthase possesses 10-CHO-THF synthetase (EC 6.3.4.3), 5,10-CH^+-THF cyclohydrolase (EC 3.5.4.9), and 5,10-CH_2-THF dehydrogenase (EC 1.5.1.5) activities. These reactions are shown in Figure 1. During the conversion of formate and glycine to serine, the coenzyme $H_4PteGlu_n$, which mediates

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[1] Abbreviations: C_1-THF synthase, C_1-tetrahydrofolate synthase; 10-CHO-THF synthetase, 10-formyltetrahydrofolate synthetase; 5,10-CH^+-THF cyclohydrolase, 5,10-methenyltetrahydrofolate cyclohydrolase; 5,10-CH_2-THF dehydrogenase, 5,10-methylenetetrahydrofolate dehydrogenase; $H_4PteGlu_n$, tetrahydropteroylglutamate containing *n* glutamyl residues; SHMT, serine hydroxymethyltransferase; c or m preceding SHMT, either the cytosolic or mitochondrial isoenzyme forms; red-cSHMT, cSHMT in which the internal ε-lysine–pyridoxal phosphate Schiff's base has been reduced with sodium cyanoborohydride; AMPPNP, 5′-adenylyl imidodiphosphate; AMPPCP, 5′-adenylyl methylene diphosphate.

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