Role of Glutamate-104 in Generating a Transition State Analogue Inhibitor at the Active Site of Cytidine Deaminase†

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ABSTRACT: The ^{19}F-NMR resonance of 5-[^{19}F]fluoropyrimidin-2-one ribonucleoside moves upfield when it is bound by wild-type cytidine deaminase from *Escherichia coli*, in agreement with UV and X-ray spectroscopic indications that this inhibitor is bound as the rare 3,4-hydrated species 5-fluoro-3,4-dihydrouridine, a transition state analogue inhibitor resembling an intermediate in direct water attack on 5-fluorocytidine. Comparison of pK_a values of model compounds indicates that the equilibrium constant for 3,4-hydration of this inhibitor in free solution is 3.5×10^{-4} M, so that the corrected dissociation constant of 5-fluoro-3,4-dihydrouridine from the wild-type enzyme is 3.9×10^{-11} M. Very different behavior is observed for a mutant enzyme in which alanine replaces Glu-104 at the active site, and k_{cat} has been reduced by a factor of 10^8. 5-[^{19}F]Fluoropyrimidin-2-one ribonucleoside is strongly fluorescent, making it possible to observe that the mutant enzyme binds this inhibitor even more tightly ($K_d = 4.4 \times 10^{-8}$ M) than does the native enzyme ($K_d = 1.1 \times 10^{-7}$ M). ^{19}F-NMR indicates, however, that the E104A mutant enzyme binds the inhibitor without modification, in a form that resembles the substrate in the ground state. These results are consistent with a major role for Glu-104, not only in stabilizing the ES‡ complex in the transition state, but also in destabilizing the ES complex in the ground state.

Cytidine deaminase (CDA)[1] catalyzes the hydrolytic deamination of various cytosine nucleosides to the corresponding uracil nucleosides (Figure 1). In *Escherichia coli*, this enzyme is a dimer of identical subunits (31 540 Da/subunit), each containing a single zinc atom (Yang et al., 1992). This bacterial enzyme accelerates the hydrolytic deamination of cytidine to uridine by nearly 12 powers of ten and has been estimated to bind the transition state for deamination with a dissociation constant of approximately 10^{-16} M (Frick et al., 1987).

Cytidine deaminase from *E. coli* is strongly inhibited by compounds resembling a hypothetical intermediate (**1,** Figure 1) that is formed by 3,4-addition of water to cytidine. The enzyme's affinity for 3,4-dihydrouridine (**3**, Figure 1), resembling **1** except for the absence of a leaving group, surpasses its affinity for product uridine by a factor of approximately 10^8. When a hydrogen atom replaces the critical 4-OH group of 3,4-dihydrouridine, enzyme affinity is reduced by a factor of approximately 10^8 (Frick et al., 1989). The crystal structure of the enzyme's inhibitory complex with the transition state analogue 5-fluoro-3,4-dihydrouridine (**5**) indicates the presence of a short hydrogen bond between the carboxyl function of glutamate-104 and the 4-OH group of the bound inhibitor (Figure 2; Betts et al., 1994). To test the possibility that an analogous hydrogen bond might play a significant role in stabilizing the hydrated substrate in the transition state for deamination, Glu-104 was replaced by Ala. Compared with the wild-type enzyme, the E104A mutant enzyme's apparent affinity for substrate cytidine had increased by a factor of 30, and its affinity for product uridine was found to have increased by a factor of 120, whereas k_{cat} had decreased by 8 orders of magnitude (Carlow et al., 1995). Thus, the carboxymethyl group of Glu-104 appears to minimize the activation barrier for deamination, not only by stabilizing the altered substrate in the transition state, but also by destabilizing the enzyme−substrate and enzyme−product complexes.

If an enzyme's catalytic activity has been severely impaired by mutation, one would expect to observe a corresponding reduction it its affinity for a transition state analogue inhibitor. When we used FZEB (**4**) to examine this possibility, we were startled to find that the E104A mutant enzyme bound the inhibitor with an affinity exceeding that of the wild-type enzyme. It became of interest to examine this interaction more closely, to determine whether the mutant enzyme binds the inhibitor, FZEB, in a form resembling cytidine in the ground state, or as the transition state analogue inhibitor 5-fluoro-3,4-dihydrouridine (**5**). This paper describes the interaction of FZEB with wild-type and mutant cytidine deaminases, using fluorescence and ^{19}F nuclear magnetic resonance spectroscopy. In separate experiments, model compounds were used to evaluate the equilibrium constant of 3,4-hydration of FZEB in free solution, allowing assessment of the actual binding affinity of the wild-type enzyme for the hydrated inhibitor, 5-fluoro-3,4-dihydrouridine (**5**).

MATERIALS AND METHODS

Enzyme and Inhibitor Preparation. Wild-type cytidine deaminase was prepared and purified according by the

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[1] Abbreviations: FZEB = 5-fluorozebularine or 5-[^{19}F]pyrimidin-2-one ribonucleoside; CDA = cytidine deaminase.

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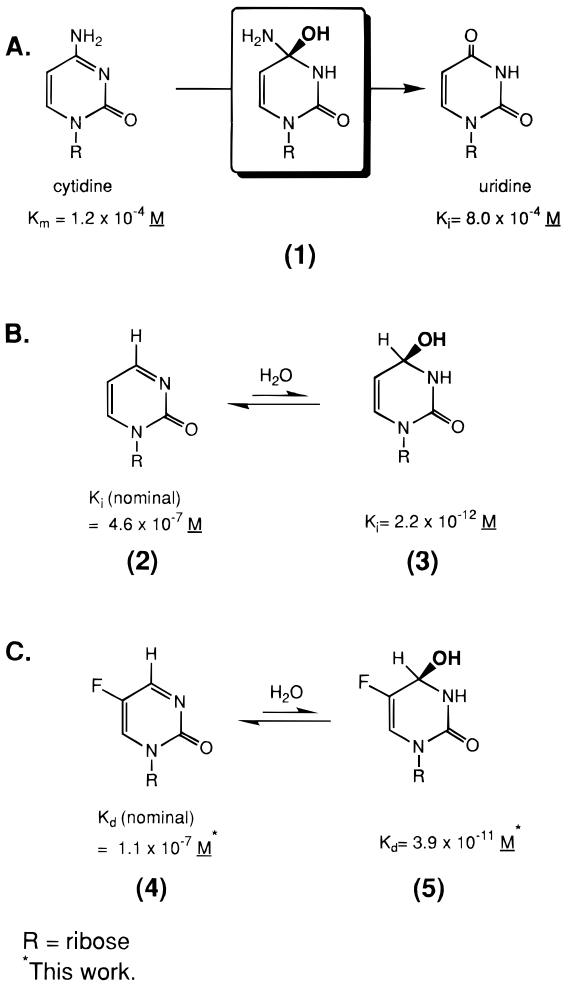

FIGURE 1: (A) Proposed mechanism of action of cytidine deaminase, involving direct water attack on cytidine to generate a tetrahedral intermediate (**1**). (B) Affinities of wild-type *E. coli* cytidine deaminase for the ground state analogue inhibitor zebularine (**2**) and the transition state analogue inhibitor 3,4-dihydrouridine (**3**).

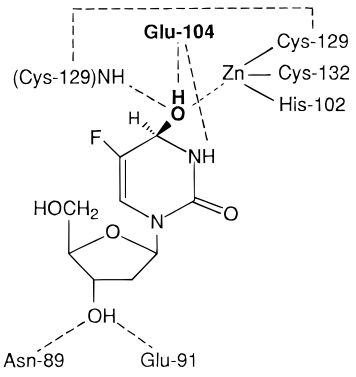

FIGURE 2: Enzyme interactions with a tetrahedral intermediate in deamination of cytidine, inferred from the crystal structure of the inhibitory complex formed between cytidine deaminase and 5-fluoro-3,4-dihydrouridine (**5**) (Betts et al., 1994).

method of Yang et al. (1992). Cytidine deaminase in which glutamate 104 had been replaced by alanine (E104A) was prepared and purified according to Carlow et al. (1995). For NMR experiments, the purified enzyme was dialyzed overnight against 25 mM potassium phosphate buffer (pH 7.0) containing 20% D_2O (v/v) and then concentrated to a volume of 0.7 mL with a Centricon-30 concentrator (Amicon). The final enzyme concentration was determined from the absorbance at 282 nm (ϵ_{282nm} = 39 000 M^{-1} cm^{-1}; Yang et al., 1992). 5-Fluorozebularine (**4**) was a gift from Dr. Victor Marquez (National Cancer Institute, NIH, Bathesda, MD).

Determination of the Dissociation Constants of 5-Fluorozebularine. Fluorescence measurements were made with a Hitachi F-2000 spectrofluorimeter. Solutions, thermostated at 25 ± 0.1°, were measured in a 3-mL cuvette (1-cm path length). The concentrations of FZEB and protein used for fluorescence analysis were maintained between 5×10^{-8} and 1×10^{-6} M, corresponding to an absorbance of 0.04 or less at the wavelength of excitation.

The fluorescence of FZEB quenched by 73% upon binding by both the wild-type and mutant proteins, but was unaffected by addition of bovine serum albumin, in potassium phosphate buffer (25 mM, pH = 7.0). To determine the dissociation constants of FZEB from these proteins, we titrated a solution of FZEB (6.6×10^{-8} M) with aliquots of enzyme in potassium phosphate buffer (25 mM, pH = 7.0). Due to the extremely tight binding of FZEB by both the mutant and wild-type proteins, it was necessary to compensate for depletion of the free enzyme. The concentration of free enzyme, $[E]_{free}$, was calculated according to the equation:

$$[E]_{free} = [E]_{total} - (\Delta F/\Delta F_{max})[FZEB]$$

where ΔF is the observed fluorescence change, ΔF_{max} is the total fluorescence quenching at saturating binding sites, and [FZEB] is the concentration of FZEB. Dissociation constants were determined by nonlinear regression analysis of a plot of the change of the relative fluorescence, ΔF, vs the concentration of free enzyme, $[E]_{free}$.

Preparation of Enzyme−Inhibitor Complexes for NMR Experiments. Inhibitory complexes used in NMR studies were formed by mixing FZEB, in small aliquots, with the enzyme solution contained in a 5 mm NMR tube. For titration of the enzyme, 0.7 mL of an enzyme solution containing 1.14×10^{-3} M enzyme subunits was placed in a 5 mm NMR tube, and the desired amount of FZEB was added from a 2×10^{-2} M stock solution.

NMR Spectroscopy. ^{19}F-NMR and ^{1}H-NMR spectra were obtained using a Varian Unity 500 NMR spectrometer. For ^{19}F-NMR, a bandwidth of 56 497 Hz was employed with a 7 μs pulse width (90°) and a repetition time of 0.64 s. Enzyme samples were prepared for NMR experiments in potassium phosphate buffer (0.025 M, pH 7.0) containing 20% D_2O for field/frequency lock. The reported pH values are uncorrected for D_2O. All experiments were performed in 5 mm NMR tubes, and the sample temperatures were controlled at 20 ± 0.1 °C. ^{19}F chemical shifts are reported in ppm with respect to 2% trifluoroacetic acid in D_2O contained in a capillary. These experiments were completed within 15 min to minimize glycosidic cleavage of FZEB (see Results).

5-Fluoro-1-methylpyrimidin-2-one (**7**). Sodium fluoromalondialdehyde[2] (200 mg, 1.8 mmol) and 1-methylurea (200 mg, 2.6 mmol) were suspended in dry ethanol (1.4 mL). Concentrated HCl (1.2 mL) was added with stirring, and a white precipitate formed almost immediately. The mixture was refluxed for 1 h. The white precipitate was filtered from the clear-yellow liquid, and the reaction was evaporated to

[2] Sodium fluoromalondialdehyde was synthesized according to the procedure of Reichard and Halbritter (1970).

dryness. Recrystallization from ethanol−ether afforded cream-colored crystals (52 mg, 22%). ^{1}H-NMR (D_2O): δ = 8.75 (s, 1H, C^4-H), 8.50 (s, 1H, C^6-H), 3.60 (s, 3H, -CH_3). ^{19}F-NMR (25 mM potassium phosphate buffer, pH 7.0, containing 20% D_2O): $\delta = -82.2$ (d, $J_{FH} = 3.45$ Hz). UV spectra (pH 2 and pH 7: λ_{max} 322 nm) and TLC mobility (R_f = 0.25 in chloroform/ethanol, 9:1 (v/v)) were identical with the values reported by Cech et al. (1977), who prepared this compound by reacting 1-methylpyrimidin-2-one with elemental fluorine in acetic acid.

5-Fluoro-1,3-dimethyl-2-oxopyrimidinium Chloride (**6′**). 1,3-Dimethylurea (200 mg, 2.2 mmol) and sodium fluoromalondialdehyde (200 mg, 1.8 mmol) were suspended in dry ethanol (1.4 mL). Concentrated HCl (1.2 mL) was added with stirring, and a white precipitate formed immediately. The reaction was refluxed for 1 h, where the contents turned slightly yellow. The precipitate was filtered off and the reaction cooled to −20 °C for 12 h. White crystals formed (60 mg, 23%) that migrated as a single spot when chromatographed on silica; R_f = 0.44 in chloroform/ethanol, 9.8:0.2 (v/v). ^{1}H-NMR (D_2O containing 0.1 M DCl): $\delta = 9.29$ (d, $J_{FH} = 2.67$ Hz, 2H, C^4 and C^6-H), 3.80 (s, 6H, 2-CH_3). ^{1}H-NMR (25 mM potassium phosphate buffer, pH = 7.0, containing 20% D_2O): $\delta = 6.29$ (d, $J_{FH} = 6.88$ Hz, 1H, C^6-H), 5.42 (d, $J_{FH} = 5.87$ Hz, 1H, C^4-H), 2.86 (s, 3H, -CH_3), 2.83 (s, 3H, -CH_3). ^{19}F-NMR (25 mM potassium phosphate buffer, pH = 7.0, containing 20% D_2O): $\delta = -83.78$ (t, $J_{FH} = 6.0$ Hz). UV spectrum, pH 1: λ_{max} 337 nm; pH 7: λ_{max} 235 nm.

RESULTS

Binding of FZEB by the E104A Mutant and Wild-Type Enzymes. This work was undertaken to explore the effect of mutating Glu-104 to Ala, which reduces k_{cat} by a factor of 10^8, on the binding of a transition state analogue inhibitor. Because catalytic activity has been reduced so profoundly in this mutant enzyme, accurate determination of a K_i value would have required extremely large quantities of mutant enzyme. Fortunately, FZEB is strongly fluorescent, and its fluorescence is quenched upon binding to either the wild-type or mutant enzyme, allowing determination of the dissociation constant of inhibitory complexes in both enzymes. This inhibitor's ^{19}F nucleus was used as an NMR probe to elucidate the structure of the enzyme-bound inhibitor.

The absorption and corrected fluorescence excitation spectra of FZEB in neutral solution are nearly identical, with a maximum at approximately 322 nm, whereas the fluorescence emission spectrum of FZEB shows a maximum at 393 nm (Figure 3). The positions of these excitation and emission maxima allow observation of FZEB−enzyme interactions without interference from other chromophores, and the fluorescence intensity is sufficient to allow detection of FZEB at a concentration as low as 1.5×10^{-8} M. Upon binding by the wild-type enzyme, the intensity of fluorescence was reduced by approximately 70% (Figure 3).

Affinities of the wild-type and mutant enzymes for FZEB were determined by observing the quenching of the fluorescence of FZEB. Figure 4 shows reductions in fluorescence intensity that were observed upon addition of wild-type and mutant enzymes. Due to the extremely tight binding of FZEB by both proteins, depletion of the added

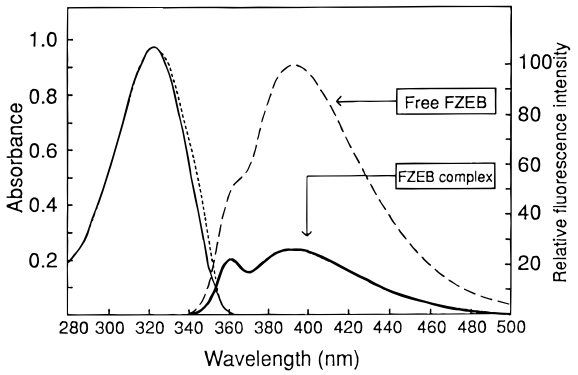

FIGURE 3: Absorption (solid line), corrected excitation (dotted line), and emission (dashed line) spectra of FZEB (**4**) in water. Concentration of FZEB for absorption was 1.4×10^{-4} M, and for fluorescence, 1×10^{-6} M. Emission spectrum of FZEB to which a saturating quantity of wild-type cytidine deaminase (3×10^{-6} M) had been added (bold line).

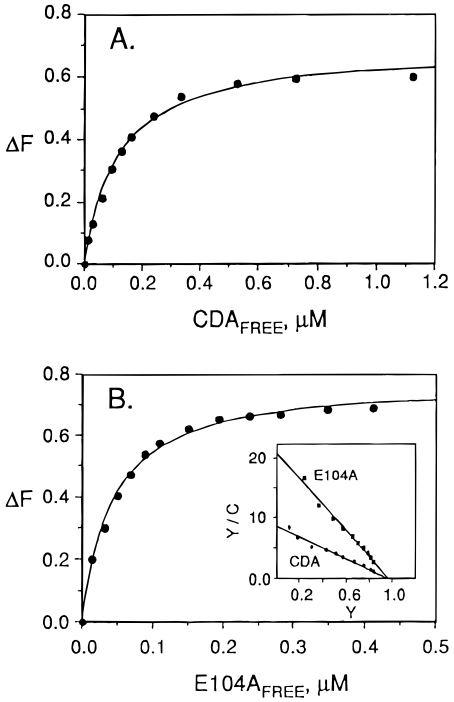

FIGURE 4: Decreases in the fluorescence intensity of FZEB (6.6×10^{-8} M) upon binding by (A) wild-type and (B) E104A mutant cytidine deaminases. The dissociation constants, calculated by nonlinear regression analysis, are 1.1×10^{-7} M for the wild-type enzyme and 4.4×10^{-8} M for the mutant enzyme. The Scatchard plot (B, insert) shows that both the mutant and wild-type enzymes have approximately one identical FZEB binding site per enzyme subunit (M_r = 31 540). *Y* is the fraction of enzyme bound to FZEB, and *C* is the concentration of free enzyme in units of μM.

enzyme could not be avoided. After correcting for the amount of ligand-bound enzyme, plots of ΔF as a function of free enzyme concentration were hyperbolic (Figure 4). Substrate cytidine ($K_{m(mutant)} = 4.2 \times 10^{-6}$ M) was found to displace FZEB from the active site of the mutant enzyme as indicated by a reappearance of fluorescence (data not shown), indicating that FZEB is bound at the mutant enzyme's active site rather than some adventitious site on the protein.

The apparent affinity of E104A for FZEB ($K_d = 4.4 \times 10^{-8}$ M) was found to have increased slightly, compared with that of wild-type enzyme (1.1×10^{-7} M). Table 1 compares these values with kinetic constants determined earlier for the wild-type and mutant enzyme. The K_d value of the wild-

Table 1: Effect of Glu-104 Replacement on Cytidine Deamination and Inhibitor Binding

	wild-type enzyme	E104A
k_{cat}/K_m (M^{-1} s^{-1})[a]	2.6×10^6	0.62
K_i, zebularine (M)[b]	4.6×10^{-7}	3.3×10^{-6}
K_d, 5-fluorozebularine (M)[c]	1.1×10^{-7}	4.4×10^{-8}

[a] Carlow et al., 1995. [b] Smith et al., 1994. [c] This work.

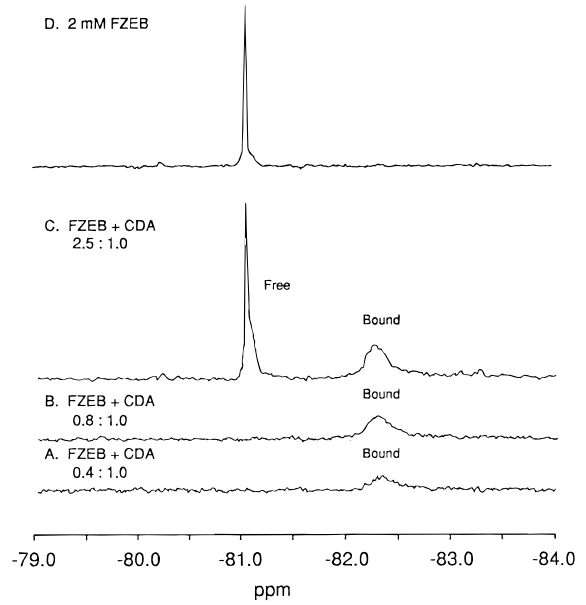

FIGURE 5: ^{19}F-NMR spectra of the titration of wild-type CDA with FZEB. All samples were in 0.025 M potassium phosphate buffer (pH 7.0) containing 20% D_2O (pH uncorrected for D_2O). (A) Spectrum of wild-type CDA (1.4×10^{-3} M) with 0.4 equiv of FZEB added (6.0×10^{-4} M). (B) Spectrum of wild-type CDA (1.4×10^{-3} M) with 0.8 equiv of FZEB added (1.1×10^{-3} M). (C) Spectrum of wild-type CDA (1.4×10^{-3} M) with 2.5 equiv of FZEB added (3.5×10^{-3} M). (D) Spectrum of FZEB (2.0×10^{-3} M).

type enzyme calculated from the present data (1.1×10^{-7} M) is in reasonable agreement with the K_i value determined earlier (1.4×10^{-7} M, Smith et al., 1994).

^{19}F-NMR Spectra of FZEB Bound by the Wild-Type and Mutant Enzymes. ^{19}F-NMR spectroscopy was used to determine the structure of FZEB bound by wild-type cytidine deaminase. A spectrum of FZEB (2 mM) alone in aqueous solution showed a doublet of doublets centered around −81.16 ppm (J_{FH} = 1.3, 4.2 Hz) (Figure 5D). Figure 5 shows spectra of samples containing different molar ratios of FZEB to wild-type CDA (1.4×10^{-3} M): (A) 0.4:1.0; (B) 0.8:1.0; and (C) 2.5:1.0. The spectrum of the first titration point (Figure 5A) contains a broad resonance centered at −82.3 ppm. As the concentration of FZEB is increased to 0.8 molar equiv, at constant concentration of enzyme, the signal intensity increases by approximately 50%. The absence of unbound FZEB at substoichiometric concentrations is consistent with tight binding of FZEB by CDA ($K_{d(nominal)} = 1.1 \times 10^{-7}$ M). When the FZEB concentration was raised to 2.5 times that of the enzyme, the broad peak representing bound FZEB increases slightly in intensity and a new sharp peak appears at −81.16 ppm, at the same position as FZEB in the control (Figure 5D). The sharpness of this latter peak suggests that it is not enzyme-associated.

Figure 6 shows ^{19}F-NMR spectra for points obtained during titration of mutant enzyme with FZEB. A spectrum

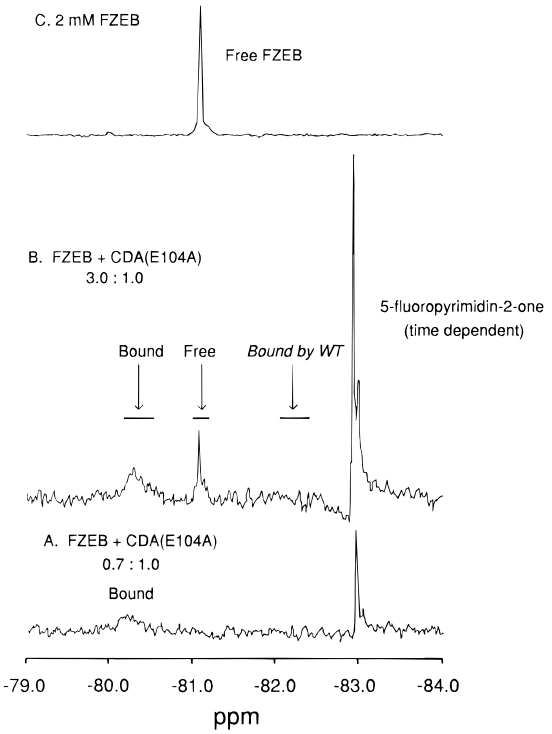

FIGURE 6: ^{19}F-NMR spectra of the titration of mutant CDA (E104A) with FZEB. All samples were in 0.025 M potassium phosphate buffer, pH 7.0, containing 20% D_2O (pH uncorrected for D_2O). (A) Spectrum of mutant CDA (E104A) (5.4×10^{-4} M) with 0.7 equiv of FZEB added (4.0×10^{-4} M). (B) Spectrum of mutant CDA (E104A) (5.4×10^{-4} M) with 3.0 equiv of FZEB added (1.6×10^{-3} M). (C) Spectrum of FZEB (2.0×10^{-3} M).

of FZEB (2 mM) alone in aqueous solution showed a doublet of doublets centered at −81.16 ppm (J_{FH} = 1.27, 4.2 Hz) (Figure 6C). Spectra A and B correspond to protein samples containing the following molar ratios of FZEB to mutant enzyme (5.4×10^{-4} M): (A) 0.7:1.0; and (B) 3.0:1.0. The spectrum from the first titration point (Figure 6A) shows a broad peak at −80.17 ppm as well as a sharp peak at −83.06 ppm. Spectrum 6B shows that the addition of excess inhibitor (3-fold) causes the intensity of the broad, protein-bound signal at −80.17 ppm to increase by approximately 20%. A sharp resonance of unbound FZEB is now apparent at −81.16 ppm. For the mutant enzyme, binding of FZEB causes a downfield shift (1.01 ppm) compared with free FZEB.

^{19}F-NMR of Model Compounds. To determine whether FZEB was bound by cytidine deaminases as its 3,4-hydrated species, a species that is extremely rare in aqueous solution (see below), a model was needed that resembles this compound in structure. The pseudobase (**6**) of 5-fluoro-1,3-dimethyl-2-oxopyrimidinium chloride (**6′**) resembles the 3,4-covalent hydrate of FZEB (**5**), except for replacement of a proton at the N-3 position by a methyl group. The pseudobase of 5-fluoro-1,3-dimethyl-2-oxopyrimidinium chloride showed a triplet centered at −83.3 ppm (J_{FH} = 6.03 Hz) (Figure 7A). The spectrum of 5-fluoro-1-methyl-pyrimidin-2-one (**7**) displayed a doublet centered around −82.29 ppm (J_{FH} = 3.45 Hz) (Figure 7B). Addition of water across the 3,4 N═C bond of the methylated model compounds is accompanied by an upfield shift in the fluorine resonance (1.01 ppm), similar in direction and magnitude to that which is observed when the fluorinated ligand is bound by the wild-type enzyme. This shift, however, is opposite

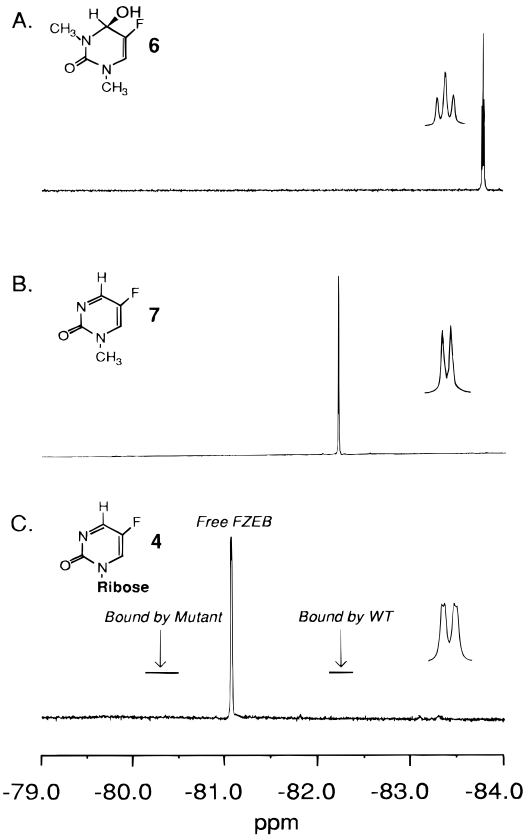

FIGURE 7: Comparison of ^{19}F-NMR spectra of fluorinated model compounds with the enzyme-bound forms. All spectra were recorded in 25 mM potassium phosphate buffer, pH 7.0, containing 20% D_2O (v/v). (A) Spectrum of the hydroxide ion adduct (**6**) of 5-fluoro-1,3-dimethyl-2-oxopyrimidinium chloride (**6′**). (B) Spectrum of 5-fluoro-1-methyl-2-pyrimidinone (**7**) (1.1×10^{-3} M) in D_2O. (C) Spectrum of FZEB (2.2×10^{-3} M) in D_2O. The positions of the signals of FZEB bound by the wild-type and mutant enzymes are shown with lines.

in direction to that observed when FZEB is bound by the mutant enzyme (Figure 7C).

Slow Glycosidic Cleavage of FZEB. During titration of E104A with FZEB, an additional signal appeared at −83.06 ppm that increased in intensity with time (Figure 6A). The signal is very narrow, suggesting that it does not represent an enzyme-associated form of the inhibitor, but rather some derivative of FZEB. As more FZEB was added (Figure 6B), the intensity corresponding to the bound form remained unchanged, while the intensities corresponding to unbound FZEB (−81.16 ppm) and to the unknown compound (−83.06 ppm) increased. A control sample of FZEB in the same buffer did not change over a much longer period of time (Figure 6C). The wild-type enzyme showed no such resonance during the same time interval. However, after the wild-type/FZEB sample was stored for 2 weeks at 4 °C, 50% of the FZEB had been converted to the new compound. Because this unknown signal does not appear to be associated with protein and is not generated in the absence of protein, it presumably arises from some product of enzyme activity. Interestingly, preparations of the largely inactive mutant enzyme appear to generate this product more rapidly than the wild-type enzyme.

To identify this degradation product, an NMR sample containing mutant enzyme (5.56×10^{-4} M) and excess FZEB (6.5×10^{-3} M) was stored for 7 days at 4 °C, after which the ^{19}F-NMR spectrum of this sample showed

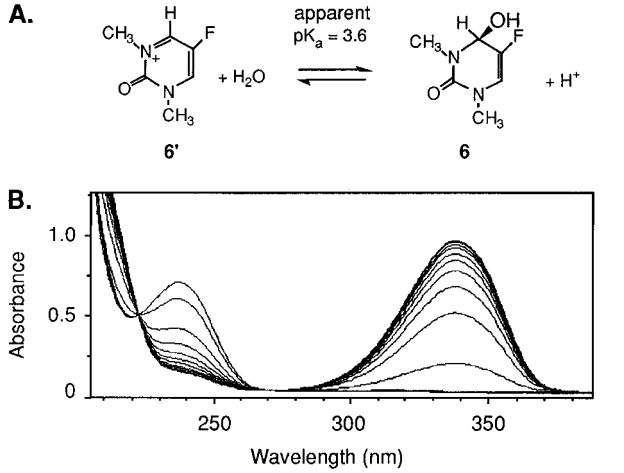

FIGURE 8: (A) Pseudobase formation of 5-fluoro-1,3-dimethyl-2-oxopyrimidinium chloride (**6**). (B) UV spectra of 5-fluoro-1,3-dimethyl-2-oxopyrimidinium chloride (II) as a function of pH, ranging from 2.0 to 5.8. The 5-fluoro-1,3-dimethyl-2-oxopyrimidinium cation has an absorption maximum at 337 nm, and its pseudobase has an absorption maximum at 235 nm. The ionic strength was maintained at 0.5 with KCl.

complete conversion of FZEB to the compound. The compound was separated from protein using a Microcon 30 apparatus (Amicon, Inc.) and purified by reverse phase high performance liquid chromatography using a Whatman Partisil 10 ODS-2 column (0.46 × 25 cm) eluted with water (0.8 mL/min). The purified compound exhibited a UV absorption spectrum (λ_{max} = 313 nm) different from that of FZEB (λ_{max} = 322 nm), but identical with that reported for 5-fluoropyrimidin-2-one (Reichardt & Halbritter, 1975), suggesting that this compound resulted from hydrolytic cleavage of the glycoside bond of FZEB. The unknown compound was identical with authentic 5-fluoropyrimidin-2-one[3] in its ^{1}H-NMR spectrum (D_2O, δ = 8.34 ppm (d, J_{FH} =1.2 Hz; C4-H and C6-H)) and its APCI mass spectrum (*m/e* 114.9; MH), recorded using a Fisons Platform device. These analytical data confirm that this compound is 5-fluoropyrimidin-2-one, generated by both the wild-type and mutant enzymes. These findings do not affect the conclusions drawn in this study, as this competing activity is slow relative to formation of the enzyme−inhibitor complex.

UV Absorption Spectra and Ionization Constants of Model Adducts. Tee and Endo (1974, 1976) established that 1,3-dimethyl-2-oxopyrimidinium cations form pseudobases. To determine the effect of substituents at the 5-position on pseudobase formation, Tee and Banerjee (1974) measured the equilibrium constants for pseudobase formation from 1,3-dimethyl-2-oxopyrimidinium chloride (pK_a = 7.0) and its 5-brominated derivative (pK_a = 3.1). In the present study the chloride salt of 5-fluoro-1,3-dimethyl-2-oxopyrimidinium cation (**6′**) was synthesized to determine the effect of 5-fluorination on pseudobase formation. The apparent equilibrium constant for combination of 5-fluoro-1,3-dimethyl-2-oxopyrimidinium chloride with hydroxide ion (K_3, Figure 10A) was determined spectrophotometrically from changes in absorption at the wavelength of maximum change as a function of pH (337 nm, Figure 8), corresponding to an equilibrium constant of 2.3×10^{10} M^{-1} for combination of

[3] Fluoropyrimidin-2-one was prepared by the method of Reichard and Halbritter (1975).

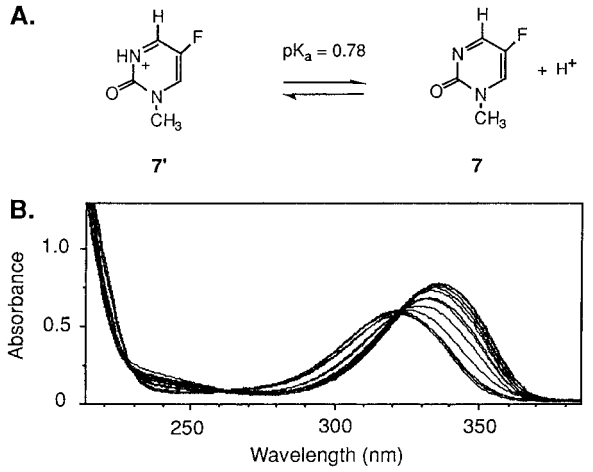

FIGURE 9: (A) N-Protonation of 5-fluoro-1-methyl-2-pyrimidinone (**7**). (B) UV spectrum of 5-fluoro-1-methyl-2-pyrimidinone (I) as a function of pH. Determinations were made at 25 °C in solutions containing hydrochloric acid in concentrations corresponding to various pH and H_o values, ranging from 0.2 to 2.0, sufficient KCl to maintain ionic strength 0.50, and 1×10^{-5} M pyrimidine. The unprotonated species has an absorption maximum at 322 nm, and the protonated compound has an absorption maximum of 336 nm.

compound **6′** with hydroxide ion. Absorption changes at this wavelength fitted theoretical titration curves as plotted by nonlinear regression using Enzfitter software (Biosoft, Cambridge, U.K.), yielding an apparent pK_a value of 3.6 ± 0.06 (Figure 8). For 5-fluoro-1-methylpyrimidin-2-one (**7**), determinations were made at 25 °C in solutions containing hydrochloric acid in concentrations corresponding to various pH and H_o values, sufficient KCl to maintain ionic strength 0.50, and 1×10^{-5} M pyrimidine. The conjugate acid of 5-fluoro-1-methylpyrimidin-2-one, presumably protonated at N-3 because of resonance stabilization (Tee & Banerjee, 1974), exhibits a pK_a value of 0.78 ± 0.02 (Figure 9).

DISCUSSION

Equilibrium Constant for Hydration of 5-Fluorozebularine in Solution, and Relative Effectiveness of ZEB and FZEB as Inhibitors. FZEB (**4**) is a strong competitive inhibitor of cytidine deaminase ($K_d = 1.1 \times 10^{-7}$ M), but is actually bound by the wild-type enzyme as its covalent hydrate, 5-fluoro-3,4-dihydrouridine (**5**), whose structure is believed to resemble that of the altered substrate in the transition state for cytidine deamination. Because 5-fluoro-3,4-dihydrouridine is extremely rare in solution, its affinity for the enzyme is correspondingly greater than the apparent K_d value of FZEB would seem to indicate (Frick et al., 1989). To determine the actual concentration of 5-fluoro-3,4-dihydrouridine that is present in solution, at the concentration of FZEB that gives half-maximal binding, we estimated the equilibrium constant for hydration as the product of the equilibrium constants for three processes: (1) ionization of water, (2) protonation of FZEB at N-3 to form its conjugate acid, and (3) addition of hydroxide ion at C-4 of the conjugate acid of FZEB (Figure 10). The last of these equilibria cannot be observed directly because of the vanishingly small concentration of hydroxide ion present at pH values low enough to protonate N-3 (conjugate acid pK_a = 2.42), but can be estimated by determining the affinity of this compound, quaternized by methylation, for hydroxide ion (Figure 10B). The quaternarized compound serves as a

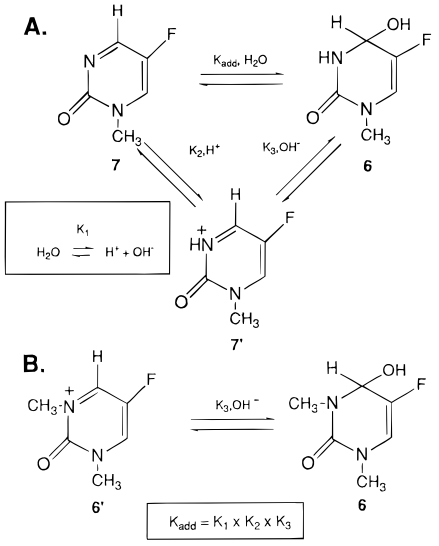

FIGURE 10: Determination of the equilibrium constant for 5-fluorozebularine (FZEB) hydration estimated from model compounds. (A) The covalent hydration of the 3,4 C═N bond is considered to proceed in three stages: (1) the dissociation of a proton from water (K_1), (2) addition of a proton to the N-3 position of FZEB (K_2), and (3) addition of hydroxide to the C-4 position of the N-3 protonated species (K_3) (Bunting, 1966; Frick et al., 1989; Jones et al., 1989). The equilibrium constant for hydration, K_{add}, is the product of these three individual equilibrium constants for these steps. (B) Values of K_3 were determined by following the spectral changes that resulted upon addition of hydroxide to the quaternary amine formed by N-methylation.

model for 5-fluoro-1-methylpyrimidin-2-one protonated at N-3 (**7′**), and its affinity for hydroxide ion (pseudobase formation) is expected to be similar (Albert, 1981; Frick et al., 1989).

The pK_a value of the conjugate acid of 5-fluoro-1-methylpyrimidin-2-one, observed spectrophotometrically at 25 °C and ionic strength 0.5, was 0.78 (Figure 9), corresponding to a value of 6.0 M^{-1} for K_2. The apparent pK_a value of the 5-fluoro-1,3-dimethyl-2-oxopyrimidinium cation (**6′**), determined spectrophotometrically under the same conditions, was 3.6, corresponding to an equilibrium constant (K_3) of 2.3×10^{10} M^{-1} for pseudobase formation. Multiplying the product of these values by the dissociation constant of water, the equilibrium constant for hydration of FZEB to form only the inhibitory *R*-isomer of 5-fluoro-3,4-dihydrouridine can be estimated as approximately 3.5×10^{-4}, using a statistical correction factor of 4 as suggested by Frick et al. (1989). From this equilibrium constant and the apparent K_d value of FZEB (1.1×10^{-7} M), we infer that the K_d value of the inhibitory *R*-isomer of 5-fluoro-3,4-dihydrouridine is approximately 3.9×10^{-11} M (Figure 11). Table 2 compares these equilibrium constants with those observed earlier for zebularine (**2**) (Frick et al., 1989), showing that the 3,4-hydrated species of FZEB (5-fluoro-3,4-dihydrouridine) is bound 18-fold less tightly than the 3,4-hydrated species of the nonfluorinated inhibitor. Because of the 70-fold more favorable equilibrium of hydration, FZEB appears, nevertheless, to be the more effective inhibitor.

Forms of 5-Fluorozebularine Bound by Wild-Type and Mutant (E104A) Cytidine Deaminases. Binding of FZEB by the wild-type enzyme is accompanied by a substantial upfield change in the chemical shift of the fluorine of FZEB (1.1 ppm; Figure 5). This change could arise from a chemical change in the inhibitor when it is bound, from an

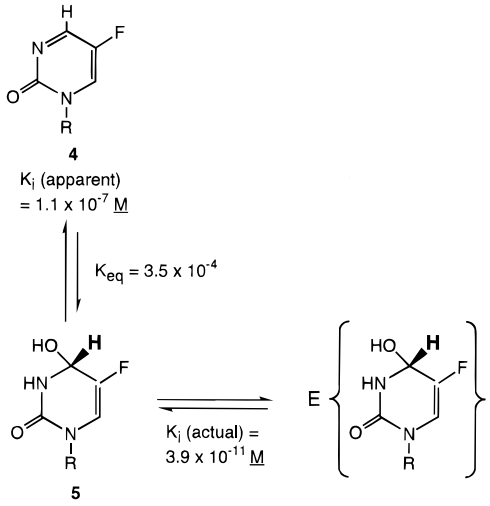

FIGURE 11: The equilibrium constant for binding of **5** by wild-type enzyme, derived from the measured K_i value of **4** for the wild-type enzyme, and the equilibrium constant for formation of **5** from **4** (see text). The actual mechanism of binding probably involves hydration at the active site, since the onset of inhibition is too rapid to be compatible with exclusive uptake of **4**.

Table 2: Comparison of the Equilibrium Constants for the Individual Steps of 3,4-Hydration of Zebularine (**2**) and 5-Fluorozebularine (**4**)[a]

	zebularine[b]	5-fluorozebularine[c]
$K_{i(apparent)}$ (M)	4.6×10^{-7} [d]	1.1×10^{-7}
K_1 (M^2)[e]	1.0×10^{-14}	1.0×10^{-14}
K_2 (M^{-1})[f]	263	6.0
K_3 (M^{-1})[g]	7.1×10^{6}	2.3×10^{10}
K_{add}[h]	4.7×10^{-6}	3.5×10^{-4}
K_i for 3,4-hydrate (M)[i]	2.2×10^{-12}	3.9×10^{-11}

[a] See Figure 10A. [b] Frick et al. (1989). [c] This work. [d] Smith et al. (1994). [e] Dissociation constant for water. [f] Equilibrium constant for protonation of 1-methylpyrimidin-2-one and 5-fluoro-1-methylpyrimidin-2-one (**7**), respectively. [g] Equilibrium constant for combination of 1,3-dimethyl-2-oxopyrimidinium cation and 5-fluoro-1,3-dimethyl-2-oxopyrimidinium cation (**6**) with hydroxide ion, respectively. [h] $K_{add} = K_1K_2K_3/4$, incorporating a statistical correction factor of 4 (see Frick et al., 1989). [i] K_i value for the inhibitory *R*-isomer of the 3,4-covalent hydrates $= K_{i(apparent)} \times K_{add}$.

altered physical environment surrounding the fluorine nucleus, or from both causes.

Results obtained in the present ^{19}F-NMR experiments are consistent with 3,4-addition of water to FZEB in its complex with the wild-type enzyme, but not in its complex with the mutant enzyme. Comparison of the NMR spectra shown in Figure 7A,B indicates that hydration of the 3,4 N═C bond of FZEB is expected to be accompanied by an upfield change (ca. 1 ppm) in the chemical shift. 5-Fluoro-1-methylpyrimidin-2-one, an analogue of the ground state form of FZEB, shows a resonance at −82.29 ppm (Figure 7B). As a model for the 3,4-covalent hydrate of FZEB, we used the pseudobase of 5-fluoro-1,3-dimethyl-2-oxopyrimidinium chloride which differs from the 3,4-covalent hydrate of FZEB in having a methyl group rather than a proton at the 3-position. The pseudobase of 5-fluoro-1,3-dimethyl-2-oxopyrimidinium chloride exhibits a resonance at −83.3 ppm, 1.0 ppm upfield from that of 5-fluoro-1-methylpyrimidin-2-one, our model of the ground state of FZEB.

Thus, binding of FZEB by the wild-type enzyme and nonenzymatic hydration of a model of FZEB are both accompanied by an upfield change in the chemical shift of the fluorine atom of the same magnitude (1.1 vs 1.0 ppm). These findings agree with the X-ray diffraction results in showing that FZEB is bound by the wild-type enzyme as the covalent hydrate 5-fluoro-3,4-dihydrouridine (Betts et al., 1994). In contrast, the fluorine signal of FZEB bound by the mutant enzyme, in which the catalytic activity is deeply depressed, is shifted in the opposite direction (Figure 6) from that expected for covalent hydration. It seems reasonable to infer that, on the mutant enzyme, bound FZEB is unhydrated, in a form that resembles a substrate in the ground state. Compared with the wild-type enzyme, the mutant enzyme shows increased affinity for FZEB, as a ground state analogue, just as the mutant enzyme shows increased affinity for substrate cytidine (Carlow et al., 1995). These results support the view that the carboxymethyl group of Glu-104 minimizes the activation barrier for deamination, not only by stabilizing the altered substrate in the transition state, but also by destabilizing the enzyme−substrate and enzyme−product complexes. The detailed structural origins of this interesting behavior remain to be elucidated.

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