Complementary Truncations of a Hydrogen Bond to Ribose Involved in Transition-State Stabilization by Cytidine Deaminase[†]

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ABSTRACT: The crystal structure of the complex formed between *Escherichia coli* cytidine deaminase and the transition-state analogue inhibitor 3,4-dihydrouridine [Betts, L., Xiang, S., Short, S. A., Wolfenden, R., & Carter, C. W. (1994) *J. Mol. Biol. 235*, 635] shows the presence of an H-bond between Glu-91 and the 3′-OH group of substituent ribose, a part of the substrate that is not directly involved in its chemical transformation. To test the contribution of this interaction to transition-state stabilization, Glu-91 was converted to alanine. The mutant enzyme is very much less active than the wild-type enzyme, with a 500-fold increase in K_m and a 32-fold reduction in k_{cat} using cytidine as substrate. No change in secondary structure is evident in the circular dichroic spectrum. As measured by k_{cat}/K_m, Glu-91 thus appears to stabilize the transition state for cytidine deamination by an overall factor of 1.7×10^4, equivalent to −5.8 kcal/mol in free energy. To test the contribution of this interaction in the opposite sense, the 3′-OH group of the substrate was replaced by a hydrogen atom. Comparing 3′-deoxycytidine with cytidine, the native enzyme shows a 17-fold increase in K_m and a 400-fold decrease in k_{cat}, indicating that the 3′-hydroxyl group of cytidine stabilizes the transition state for deamination by an overall factor of 6.3×10^3, equivalent to −5.2 kcal/mol in free energy, as measured by k_{cat}/K_m. After one binding partner has been removed, however, the effect of removing the remaining partner is relatively slight. For the mutant enzyme E91A, removal of the 3′-hydroxyl group from substrate cytidine reduces k_{cat}/K_m by a factor of only 3. Complete removal of substituent ribose reduces the wild-type enzyme's k_{cat}/K_m by a factor of more than 10^8; thus, substituent ribose, although distant from the site of chemical transformation of the substrate, contributes at least 11 kcal to the free energy of stabilization of the transition state for cytidine deamination, matching the apparent contribution to transition state binding made by the 4-OH group of the pyrimidine ring, which is at the site of substrate transformation [Frick, L., Yang, C., Marquez, V. E., & Wolfenden, R. (1989) *Biochemistry 28*, 9423].

Cytidine deaminase from *Escherichia coli* exemplifies a group of enzymes that are involved in the metabolism of nucleosides and in the posttranslational modification of mRNA. Many features of the catalytic mechanism of this zinc-dependent enzyme are understood to arise from its ability to stabilize a tetrahedral-like transition state in hydrolytic deamination, formed by 3,4-addition of water to cytidine (Figure 1a) (*1*−*7*). The 4-OH group of this species, derived from the attacking water molecule, contributes a factor of approximately 10^8-fold to the enzyme's affinity for the altered substrate in the transition state, as indicated by earlier experiments involving truncation of a transition-state analogue or mutation to alanine of a glutamic acid residue (Glu-104) at the active site (*2*, *9*).

The specificities of enzymes, with respect to parts of a substrate that are separated by some distance from the site of its chemical transformation, are of theoretical and practical interest. The present paper explores the contribution to transition-state stabilization made by a part of substrate cytidine that is not directly involved in its chemical transformation: substituent ribose. The crystal structure of the complex formed between *E. coli* cytidine deaminase and the transition-state analogue inhibitor 3,4-dihydrouridine indicates, in addition to the above interactions that are directly involved in substrate transformation, the presence of a charged H-bond between Glu-91 and the 3′-hydroxyl group of the substrate's substituent ribose (Figure 1b) (*1*). This paper describes an investigation of the role of this interaction in substrate binding and catalysis by two methods: mutagenesis of the side chain of Glu-91 and removal of the 3′-OH group from the substrate. In addition, both of these deletions were combined. We have also examined the activities of both the native and mutant enzymes on cytosine from which the glycoside substituent has been removed completely.

EXPERIMENTAL PROCEDURES

Materials. Nucleosides were purchased from Sigma Chemical Co. Other reagents were analytical-grade, and were used without further purification.

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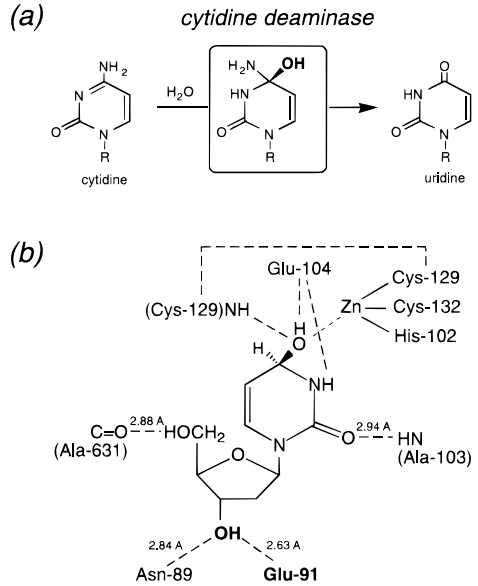

FIGURE 1: (a) Reaction catalyzed by cytidine deaminase. The proposed mechanism of catalysis involves direct water attack on cytidine to generate a tetrahedral intermediate (boxed). (b) Enzyme interactions with the tetrahedral intermediate in deamination of cytidine, inferred from the crystal structure of the inhibitory complex formed between cytidine deaminase and the transition-state analogue 3,4-dihydrouridine (*7*). The 2′-hydroxyl group, which makes no significant contribution to binding or catalysis (*8*), has been omitted for clarity.

Recombinant DNA Procedures. The subcloning and sequencing of the *cdd* gene construct was performed as described by Smith et al. (*10*). Genes encoding wild-type and mutant protein, along with the necessary upstream promoter/operator sequences, were derived from pBR322 plasmids. After mutagenesis of the wild-type *cdd* gene, the region of the mutant gene containing the codon change was verified by nucleotide sequence determination and subcloned as a *Nsi*I−*Bgl*II-ended DNA fragment into unmutagenized and *Nsi*I−*Bgl*II digested wild-type plasmid pAA5970. This procedure ensured that only the desired mutations were present in the final clone used for enzyme expression.

Enzyme Purification. Plasmids harboring the wild-type *cdd* gene and the E91A mutant allele were transformed into *E. coli* SS6130 (*cytR* Δ*cdd*) for protein production, as described by Smith et al. (*10*). In this strain, transcription of the plasmid-borne *cdd* gene is completely derepressed, and cytidine deaminase is expressed only from the plasmid-borne gene. Bacterial cultures were grown for 16−18 h at 37 °C in Luria broth containing ampicillin (100 μg/mL), recovered by centrifugation (3 g wet weight), and suspended in buffer (12 mL) containing Tris-HCl (50 mM, pH 7.5), KCl (50 mM), and glycerol (10% v/v). Cell extracts were prepared by passage of this material through a French press (2 × 11 000 psi) Intact cells and cell debris were removed by centrifugation (15 000 rpm for 15 min in an SS-34 rotor), and the crude supernatant was centrifuged at 100 000*g* for 1 h. The resulting supernatant (portions not exceeding 25 mg of total protein) was then applied to an anion-exchange column (Poros 20 HQ, 0.46 × 10 cm, Perspective Biosystems, Cambridge, MA) equilibrated with the same buffer and developed at a flow rate of 10 mL/min. At least 90% of the enzyme was recovered in the flowthrough fraction. This protein was dialyzed against Tris-HCl (50 mM, pH 7.5) containing glycerol (10% v/v) to remove KCl. This material, in portions not exceeding 25 mg of total protein, was applied to the same column equilibrated in the same buffer. When the column was developed with a gradient of increasing KCl, cytidine deaminase emerged at a salt concentration of 0.15 M KCl. Analysis by SDS−PAGE indicated that the enzyme was at least 95% homogeneous. The concentration of purified wild-type and mutant enzyme was determined from the absorbance at 280 nm, using an extinction coefficient of 39 000 M^{-1} cm^{-1} (*4*).

Enzyme Assays. The cytidine deaminase activity of the wild-type and mutant enzyme on cytidine and 3′-deoxycytidine was monitored by following the change in UV absorbance when the cytosine nucleoside was converted to the corresponding uracil nucleoside (*11*). All assays were performed in Tris-HCl buffer (50 mM, pH 7.5) at 25 °C in quartz cuvettes (0.2 cm path length). For the wild-type enzyme, 3′-deoxycytidine deamination was monitored at 298 nm ($\Delta\epsilon = -179$ M^{-1} cm^{-1}). The concentration of 3′-deoxycytidine was varied from 0.1 to 10.0 mM, and the concentration of enzyme in the cuvette was 5 μM. For the E91A mutant enzyme, both cytidine and 3′-deoxycytidine deamination was monitored at 301 nm ($\Delta\epsilon = -71$ M^{-1} cm^{-1}). This high wavelength allowed for concentrations of greater than 0.1 M for both nucleosides in the assay, and the relatively large change in absorbance upon deamination allowed for precise kinetic measurements. The concentration of mutant enzyme was maintained at 2.9 μM for the cytidine reactions and 5.8 μM for the 3′-deoxycytidine reactions. At these enzyme concentrations the reactions were typically complete within 20 min. Kinetic constants were obtained by nonlinear regression analysis using the computer program Enzfitter (Biosoft, Cambridge, U.K.).

Cytosine Deamination. Reaction mixtures (100 μL) contained enzyme (0.1 mM either wild-type or mutant enzyme), cytosine (1−40 mM), and Tris-HCl (50 mM, pH 7.5) and were maintained at 25 ± 0.1 °C. At timed intervals ranging from 1 to 90 h, aliquots (5 μL) were removed and diluted 200-fold into reaction buffer (995 μL) in a quartz cuvette (1 cm), and the UV spectra were recorded. The rate constants for conversion of cytosine to uracil were calculated from the change in absorbance at 259 nm ($\Delta\epsilon = 8200$ M^{-1} cm^{-1}). To determine whether the observed cytosine deaminating activity was due to cytidine deaminase or to a contaminating enzyme, we also performed these same reactions in the presence of the potent cytidine deaminase inhibitors tetrahydrouridine (1.5×10^{-6} M) and pyrimidin-2-one ribonucleoside (3×10^{-6} M), concentrations 10-fold in excess of their respective K_i values (*2*).

Zinc Analysis. Samples of enzyme were analyzed for zinc content using an Instrumentation Laboratories S-12 flame atomic absorption spectrometer, as described previously (*10*).

Circular Dichroism Spectroscopy. CD spectroscopy was used to compare the overall secondary structure of wild type with the E91A mutant protein. The proteins were dialyzed against 20 mM potassium phosphate buffer, pH 7.0, and their concentration was adjusted to 10 μM. CD was measured with an Avis 60 DS spectrometer. Each spectrum was recorded in 0.5 nm wavelength increments and was normalized for protein concentration, and the observed ellipticity, θ, was background-corrected against the spectrum obtained for the dialysis buffer.

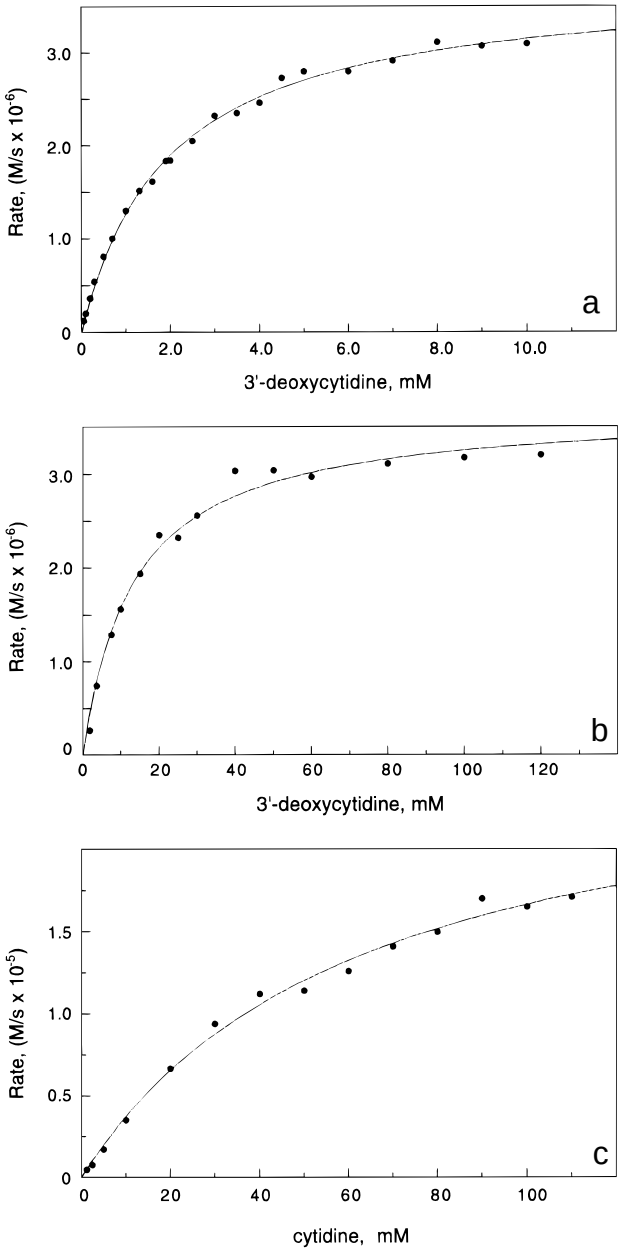

FIGURE 2: (a) Action of wild-type cytidine deaminase (5 μM subunits) on 3′-deoxycytidine; (b) action of the E91A mutant enzyme (2.9 μM subunits) on cytidine; (c) action of the E91A mutant enzyme (5.8 μM subunits) on 3′-deoxycytidine. Activity is shown as a function of substrate concentration. The individual reactions were monitored as described in the text.

RESULTS

Deamination of 3′-Deoxycytidine by Wild-Type Cytidine Deaminase. Figure 2 and Table 1 show that the wild-type enzyme deaminates 3′-deoxycytidine much less efficiently than it deaminates cytidine. Compared with cytidine, the value of k_{cat} for 3′-deoxycytidine is reduced by a factor of 374 and the apparent binding affinity, K_m, increases by a factor of 17. The value of k_{cat}/K_m is therefore reduced by a factor of approximately 6.3×10^3 for 3′-deoxycytidine, equivalent to 5.2 kcal/mol in free energy.

Preparation and Properties of Cytidine Deaminase Mutant E91A. After the site-specific change of Glu-91 to Ala had been introduced into the *E. coli cdd* gene, the presence of the mutation (GAA → GCG) was verified, and the variant

Table 1: Kinetic Parameters of Wild-Type and E91A Cytidine Deaminases[a]

	substrate	
	cytidine	3′-deoxycytidine
	wild-type enzyme	
K_m (mM)	0.120	2.0
k_{cat} (s^{-1})	299	0.8
k_{cat}/K_m (M^{-1} s^{-1})	2.5×10^6	400
	E91A enzyme	
K_m (mM)	62	13
k_{cat} (s^{-1})	9.3	0.6
k_{cat}/K_m (M^{-1} s^{-1})	150	46

[a] The kinetic parameters for the wild-type enzyme with cytidine as substrate are from ref *8*. Standard errors in the present work were less than or equal to ±11% for K_m values and ±5% for k_{cat} values.

was expressed as described for earlier mutants of *E. coli* cytidine deaminase (*9*, *10*, *12*). As in the case of those mutant proteins, the CD spectrum of the purified E91A mutant protein (not shown) was found to be indistinguishable from that of the wild-type enzyme, indicating the structural integrity of the mutant enzyme. After exhaustive dialysis against a "metal-free" buffer, the variant was found to contain 0.85 (± 0.1) zinc atom per subunit (31 540 Da), identical with that obtained for the wild-type enzyme (within experimental error). This provided further evidence of the structural integrity of the active site of the mutant enzyme.

Effect of Glu-91 Replacement on Cytidine Deamination. By monitoring the change in absorbance at 301 nm in short path length cuvettes (0.2 cm), we were able to monitor deamination continuously at concentrations of nucleosides as high as 120 mM. The data in Figure 2b show the rate of deamination of cytidine as a function of cytidine concentration for the E91A variant. Even at a cytidine concentration as high as 110 mM, the E91A variant is not saturated with substrate. Further increases in cytidine concentration were not possible because of its limited solubility in aqueous solutions at the assay temperature. When the data were subjected to nonlinear regression analysis using the computer program Enzfitter, the fit of the data to a theoretical curve was close enough to allow values for k_{cat} and K_m to be calculated within a reasonable margin of error (Table 1), and a K_m value of 62 mM was obtained. Table 1 shows that when alanine replaces Glu-91, the value of k_{cat} is reduced by a factor of 32 and the mutant enzyme's K_m value for cytidine has increased by a factor of 520. This corresponds to a 1.7×10^4-fold reduction in k_{cat}/K_m. Thus, by its presence in the wild-type enzyme, the side chain of Glu-91 stabilizes the transition state for cytidine deamination by 5.8 kcal/mol in free energy.

Effect of Glu-91 Replacement on the Deamination of 3′-Deoxycytidine. Figure 2c describes the deamination of 3′-deoxycytidine by the E91A mutant enzyme. Relative to catalysis of 3′-deoxycytidine deamination by the wild-type enzyme, the k_{cat} value decreases slightly (−25%) and the value of K_m increases by a factor of 6.5 (Table 1). By its presence in the wild-type enzyme, the side chain of Glu-91 appears to stabilize the transition state for 3′-deoxycytidine deamination by a factor of 8.7, equivalent to 1.3 kcal/mol in free energy.

Effect of the 3′-OH Group of Cytidine on Its Deamination by Wild-Type Enzyme and Variant E91A. The data sum-

marized in Table 1 can be viewed in a different way. Relative to its action on cytidine, the wild-type enzyme exhibits a k_{cat}/K_m value for 3′-deoxycytidine that is reduced by a factor of 1.6×10^4. The mutant enzyme presents a sharp contrast. Relative to its action on cytidine, variant E91A exhibits a k_{cat}/K_m value for 3′-deoxycytidine that is reduced by a factor of only 3.2, equivalent to 0.7 kcal/mol in free energy.

Testing the Ability of Cytidine Deaminase To Act on Cytosine. In the presence of very high concentrations of wild-type enzyme (0.1 mM in subunits), cytosine was deaminated very slowly to uracil, in a reaction that could be observed by spectrophotometry and HPLC after very long reaction times. The rate of this reaction showed no signs of saturation with cytosine, at concentrations near its solubility limit (4×10^{-2} M), so that values of k_{cat} and K_m could not be determined. However, the slopes of plots of pseudo-first-order rate constants as a function of enzyme concentration yielded an apparent value of 0.025 M^{-1} s^{-1} for k_{cat}/K_m. Comparison of this value with the value observed for cytidine (Table 1) seemed at first to indicate that k_{cat}/K_m decreases by a factor of 10^8 when substituent ribose is removed.

Further experiments indicate that even this extremely low activity represents an overestimate of cytidine deaminase's activity on the aglycon. We are grateful to a reviewer for suggesting that the enzyme's apparent cytosine deaminating activity, low as it was, might be due to the presence of a contaminating enzyme rather than to cytidine deaminase itself. We observed that in the presence of tetrahydrouridine and pyrimidin-2-one ribonucleoside, two potent competitive inhibitors of cytidine deaminase, the rate of deamination of cytosine was unaffected, even when both of these inhibitors were tested at concentrations 10-fold in excess of their respective K_i values. The existence of this trace ($1/10^8$) of contaminating cytosine-deaminating activity, whatever its nature may be, implies that the true ratio of k_{cat}/K_m values for cytidine deaminase, acting on cytidine vs cytosine, is even higher than 10^8-fold.

It seems reasonable to inquire whether this drastic reduction in activity might be due to differences in inherent reactivity that may exist between cytosine and cytidine. The somewhat lower pK_a value of cytidine (4.22) than that of cytosine (4.60) (*13*) suggests that substituent ribose is somewhat electron-withdrawing, activating cytidine for nucleophilic attack compared with cytosine. Recent experiments indicate that the uncatalyzed rate of cytosine deamination occurs approximately 3-fold more slowly (Carlow, Ridgway, Wolfenden, unpublished results) than that of cytidine (*3*). These observations indicate that the contribution of substrate ribose to the intrinsic stability of cytidine is modest.

DISCUSSION

These experiments on cytidine deaminase provide an indication of the contributions made to catalysis by interactions of a substituent (ribose) and a functional group (the 3′-hydroxyl) that are distant from the site of chemical transformation of the substrate and exert a negligible influence on the substrate's inherent chemical reactivity. Such effects have been encountered before [see, for example, a discussion by Koshland (*14*)].

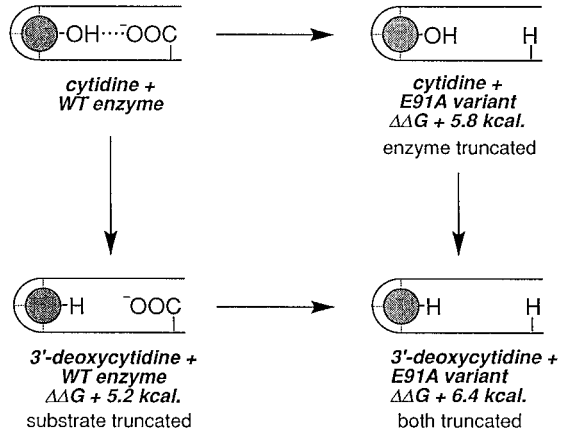

FIGURE 3: Effects of removing a ribose−enzyme interaction by truncating the enzyme, the substrate, or both. Elimination of either interaction produces almost as much effect as elimination of both. This diagram ignores possible contributions of small conformation changes and of solvent water (see text).

For the H-bond between Glu-91 and the substrate's 3′-OH group, similar changes in the free energy of transition-state stabilization are found to result from truncation of binding partners contributed by either the active site or the ligand (Figure 3). Thus, conversion of Glu-91 to alanine reduces k_{cat}/K_m for cytidine by a factor of 1.7×10^4, equivalent to a change of 5.8 kcal/mol in the free energy of transition-state binding. For the wild-type enzyme, but not for variant E91A, removal of the substrate's 3′-OH group reduces k_{cat}/K_m by a similar factor (6.3×10^3), equivalent to a change of 5.2 kcal/mol in the free energy of transition-state binding. The rough agreement between these results suggests that the contribution to transition-state binding made by the 3′-OH group's H-bond to Asn-89 (Figure 1b) may be relatively minor, consistent with the fact that this H-bond is longer than the 3′-OH group's H-bond to Glu-91 and does not involve a charged group.

This simple analysis ignores the potential role of solvent water and of changes in enzyme conformation that escape detection by circular dichroism measurements as determinants of binding affinity. The potential "leveling effect" of solvent interactions, in comparisons of this kind, has been considered in some detail elsewhere (*15, 16*). The crystal structures of these new ligand complexes of cytidine deaminase should be helpful in analyzing possible contributions from these effects.

The very large effect of completely removing ribose from cytidine, on its activity as a substrate for the wild-type enzyme ($\sim 10^8$-fold in k_{cat}/K_m), has been determined only as a lower limit. The magnitude of this effect appears especially startling if one considers that cytosine and cytidine are similar in intrinsic reactivity and that cytosine should presumably be able to enter any active site that can bind cytidine. Substituent ribose, although distant from the site of chemical transformation of the substrate and not involved in its intrinsic reactivity, contributes more than 11 kcal to the free energy of stabilization of the transition state for cytidine deamination, matching the apparent contribution to transition state binding made by the 4-OH group on the pyrimidine ring (*2*). Cytidine deaminase appears to act as a kind of "chelating agent", completely surrounding the altered substrate in the transition state (*1*), in a way that may allow the

enzyme to exploit every possible binding contact to stabilize this species as suggested earlier (*17*). In arriving at such a scenario, enzymes acting on nucleosides and nucleotides may enjoy a special advantage, in that the various parts of the substrate occupy a relative fixed relation to each other in space, tending to reduce the entropic costs of binding such an "irrelevant" substituent as ribose.

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