# Transition-State Selectivity for a Single Hydroxyl Group during Catalysis by Cytidine Deaminase†

Shibin Xiang,‡ Steven A. Short,§ Richard Wolfenden,‡ and Charles W. Carter, Jr.*,‡

*Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7260, and Division of Experimental Therapy, Wellcome Research Laboratory, Research Triangle Park, North Carolina 27709*

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ABSTRACT: Cytidine deaminase binds transition-state analog inhibitors $\sim 10^7$ times more tightly than corresponding 3,4-dihydro analogs containing a proton in place of the 4-hydroxyl group. X-ray crystal structures of complexes with the two matched inhibitors differ only near a "trapped" water molecule in the complex with the 3,4-dihydro analog, where contacts are substantially less favorable than those with the hydroxyl group of the transition-state analog. The hydrogen bond between the hydroxyl group and the Glu104 carboxylate shortens in that complex, and may become a "low-barrier" hydrogen bond, since at the same time the bond between zinc and the Cys132 thiolate ligand lengthens. These differences must therefore account for most of the differential binding affinity related to catalysis. Moreover, the trapped water molecule retains some of the binding energy stabilizing the hydroxyl group in the transition-state analog complex. To this extent, the ratio of binding affinities for the two compounds is smaller than the true contribution of the hydroxyl group, a conclusion with significant bearing on interpreting difference free energies derived from substituent effects arising from chemical modification and/or mutagenesis.

Cytidine deaminase (CDA:[1] Cohen & Wolfenden, 1971; Ashley & Bartlett, 1984; Frick et al., 1989) and adenosine deaminase (ADA; Kati & Wolfenden, 1989b) both catalyze reactions in which a zinc-activated hydroxide ion attacks the purine or pyrimidine to form a tetrahedral transition state which is bound with dissociation constants of roughly $10^{-16}$ M. Much of this affinity has been realized in stable transition-state analog inhibitors related to cytidine by modification of the C4 substituent (Figure 1a). The cytidine deaminase transition-state analog zebularine 3,4-hydrate, or ZEB−$H_2O$, has a $K_i$ of $1.2 \times 10^{-12}$ M, whereas the dihydro analog, 3,4-dihydrozebularine, or DHZ, has a $K_i$ of $3 \times 10^{-5}$ M (Frick et al., 1989). These two nucleosides and their interactions with the enzyme are illustrated schematically in Figure 1b. They are stereochemically equivalent except for replacement of the hydroxyl group on C-4 by a hydrogen atom. The hydroxyl group involved in this substituent effect is analogous to that which develops in the tetrahedral transition state. It contributes 10.1 kcal/mol to the observed differential binding energy of ZEB−$H_2O$, relative to that of DHZ, and hence at least this much to the transition-state affinity. In a comparable case, adenosine deaminase binds 6-hydroxy-1,6-dihydropurine ribonucleoside $10^7$-fold more tightly than 1,6-dihydropurine ribonucleoside (Wolfenden et al., 1967; Wolfenden, 1969).

The structural basis of this unusually large substituent effect is of particular interest because the hydroxyl group is the most conspicuous structural feature that distinguishes the altered substrate in the transition state from the substrate in the ground state (Wolfenden & Kati, 1991). Our X-ray crystal structure of a fluorinated transition-state analog, 5-fluorozebularine, complexed with CDA (CDA·FZEB; Betts et al., 1994) revealed that, in the active site of CDA, a zinc atom is coordinated in a tetrahedral ligand field to cysteines 129 and 132 and histidine 102, plus the C4 hydroxyl group of the inhibitor, confirming that FZEB binds to CDA as a hydrate, FZEB−$H_2O$. However, as illustrated in Figure 1b, one could not unambiguously attribute the strong differential binding affinity to interactions with the single oxygen atom without answering additional questions about the structures of exactly paired inhibitors: (1) Is a zinc-bound water molecule also present in the CDA·DHZ complex? (2) Does any change in either enzyme or ligand conformation occur between the two complexes CDA·DHZ and CDA·ZEB−$H_2O$? (3) Do the inhibitors DHZ and ZEB−$H_2O$ make comparable interactions with the enzyme elsewhere?

We report here new X-ray crystal structures of the enzyme complexed with ZEB−$H_2O$ and DHZ, which now provide direct answers to these questions, clarifying and underscoring the central importance of interactions with the hydroxyl group to the CDA catalytic mechanism. By analogy with the structures of the CDA·FZEB−$H_2O$ (Betts et al., 1994) and adenosine deaminase−nebularine hydrate complexes (Wilson et al., 1991), we find that the transition-state analog ZEB binds to CDA in its hydrated form, ZEB−$H_2O$ (Frick et al., 1989). In the complex with DHZ we find that a zinc-bound water molecule is "trapped" at the active site. The two crystal structures differ only in the immediate vicinity of where bonds are being made and broken during the catalyzed

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* Author to whom correspondence should be addressed: telephone (919) 966-3263; FAX, (919) 966-2852; E-mail, carter@med.unc.edu.

‡ University of North Carolina at Chapel Hill.

§ Wellcome Research Laboratory.

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[1] Abbreviations: CDA, cytidine deaminase; ADA, adenosine deaminase; ZEB, zebularine or pyrimidin-2-one riboside; ZEB−$H_2O$, 3,4-hydrated zebularine; DHZ, 3,4-dihydrozebularine.

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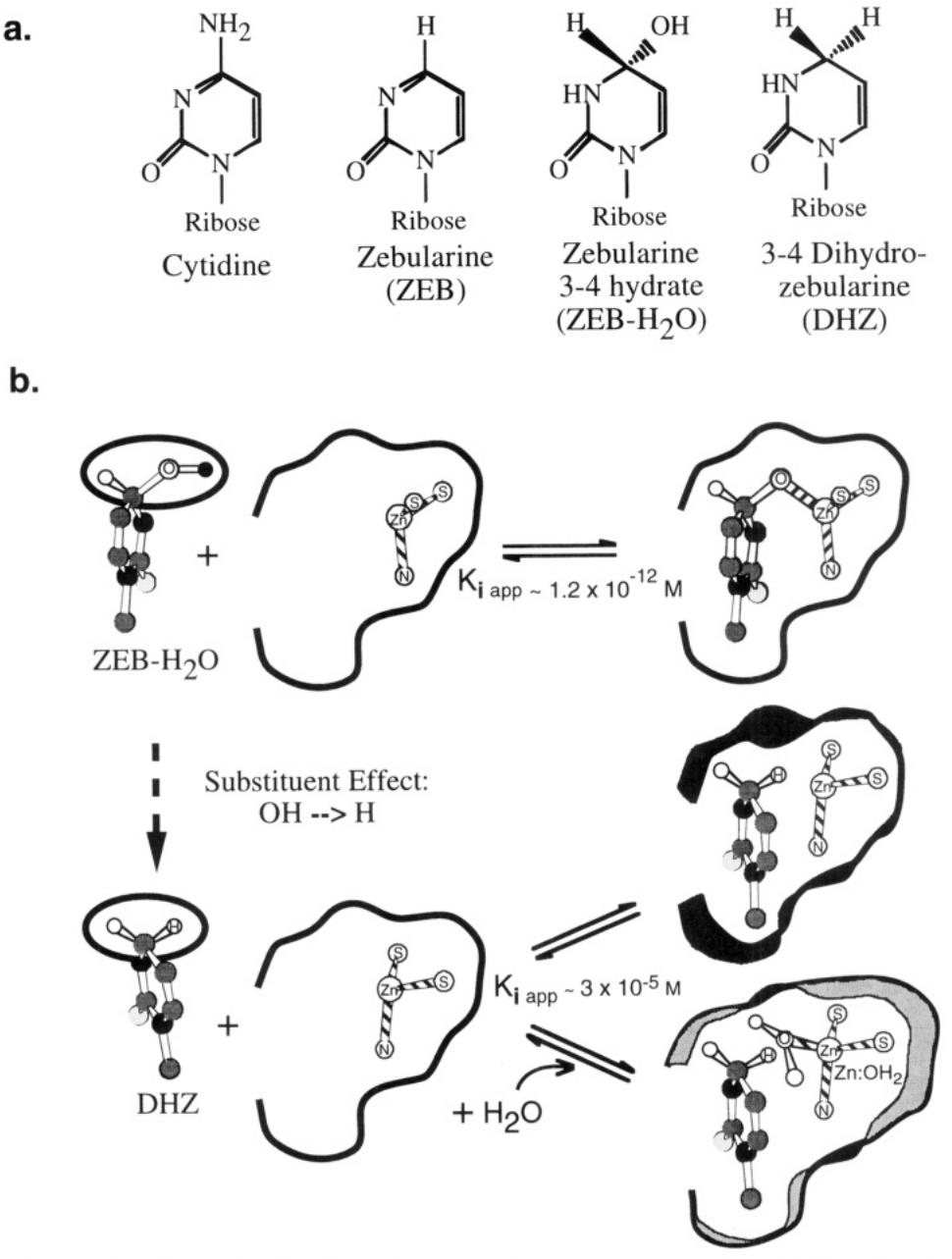


FIGURE 1: (a) Pyrimidine nucleosides involved in this work, compared to the substrate, cytidine. (b) Formal binding equilibria for the complexes CDA:ZEB−$H_2O$ and CDA:DHZ. Substitution of the hydroxyl group by hydrogen leads to inhibition constants for the two compounds which differ by 7 orders of magnitude, equivalent to ~10 kcal/mol. *A priori*, DHZ might be expected to bind to a site illustrated by the bold line, in which the zinc-bound water molecule is either absent or present. In the former case, the active site might collapse around the ligand (dark shading), relative to its configuration in the transition-state analog complex with ZEB−$H_2O$ (light lines). The latter alternative is equivalent to "trapping" a water molecule in the active site, which might lead to an increase in the size of the active site cavity (light shading).

reaction. Changes in binding affinity appear to result both from unfavorable van der Waals interactions between DHZ and the trapped water molecule and from strengthened hydrogen-bonding interactions with the ZEB−$H_2O$ hydroxyl group.

## EXPERIMENTAL PROCEDURES

*Crystallographic Data.* Both zebularine and 3,4-dihydrozebularine were kindly provided by V. E. Marquez. Crystals of CDA·DHZ and CDA·ZEB−$H_2O$ were grown by vapor diffusion in hanging drops initially containing 14−20% saturated ammonium sulfate (pH 6.2) and 11−16 mg/mL protein previously equilibrated with the inhibitors against 1.0−1.6-mL reservoirs of 32−45% saturated ammonium sulfate at 4 °C. Crystals of both complexes are isomorphous to those of CDA·FZEB (Betts et al., 1994), having space group $P3_121$ and unit cell dimensions of $a = b = 120.3$ Å, $c = 78.4$ Å, and $\beta = 120°$. Data were collected at room temperature using an RAXIS II image plate and Rigaku RU-200 rotating anode. The CDA·FZEB model with FZEB deleted was used to initiate refinements of both CDA·DHZ and CDA·ZEB, which were carried out first with X-PLOR (Brunger et al., 1987) and then with TNT (Tronrud et al., 1987). Zinc, zinc ligands, and inhibitors were also omitted in the first cycle of X-PLOR refinement, in order to reduce model bias, and then rebuilt into the model on the basis of the $\{|F_o| - |F_c|, \phi_c\}$ map before the second refinement cycle. Between refinements, the model was rebuilt from both $\{2|F_o|$

Table 1: Crystallographic Data for the CDA·ZEB and CDA·DHZ Complexes

| | CDA·ZEB | CDA·DHZ |
|---|---|---|
| data | | |
| resolution limit (Å) | 2.3 | 2.1 |
| no. of observations | 72498 | 118087 |
| no. of unique reflections | 24211 | 34000 |
| completeness (%) | 80 | 85 |
| $R_{merge}$ (%) | 8.2 | 7.81 |
| refinement | | |
| protein atoms | 2220 | 2220 |
| ligand atoms | 16 | 15 |
| zinc atom | 1 | 1 |
| ordered waters | 48 | 49 |
| rms bond length deviation (Å) | 0.01 | 0.01 |
| rms bond angle deviation (deg) | 2.4 | 2.4 |
| *R*-factor | 0.19 | 0.19 |

$-|F_c|, \phi_c\}$ and $\{|F_o| - |F_c|, \phi_c\}$ maps using FRODO (Jones, 1985). Water molecules were added after the *R*-factor at 2.3 Å resolution reached a value less than 0.23 and manual rebuilding would not improve the matches between the map and the model (Table 1).

*Refinement.* Refinement of ligand complexes to X-ray amplitude constraints is still not a routine procedure, in part because the energy terms required to impose stereochemical constraints are poorly developed. We found that particular attention had to be paid to the behavior of groups near the active site and that considerable manual intervention was required to eliminate features from the residual $\{|F_o| - |F_c|, \phi_c\}$ difference Fourier map. Thus, during the course of refinement stereochemical restraints for active site groups, including the zinc−ligand distances, were modified empirically, by small amounts, to minimize peaks and holes around the active site in the difference map.

It should be noted that the surprising C4−O4 distances in the two complexes resulted in spite of the restraints imposed during refinement. In particular, refinement of the hydroxyl group in the ZEB−$H_2O$ complex was restrained initially to a target covalent bond distance of 1.43 Å, adjusted ultimately to 1.60 Å, and the refinement of the water molecule in the DHZ complex was restrained throughout the refinement by a repulsive van der Waals interaction based on an interatomic distance of 2.8 Å. The refined positions that minimized the features of the difference Fourier maps nevertheless involved a long C4−O4 distance of $1.6_6$ Å in the former case and a short distance of $2.4_8$ Å in the latter case.

*Coordinate Errors.* The rms error in atomic coordinates estimated from a Luzzati plot (Luzzati, 1952) was 0.25 Å for both structures. This estimate may be high for the atoms at the active site, which have very low *B*-factors. As noted below, comparison between independently refined structures of several different CDA complexes provides an estimate of ~0.15 Å for the rms deviation of backbone atoms, and the atoms at the active site are probably better defined than the overall main chain. Moreover, our conclusions derive not only from the refined coordinates but also from features in $\{|F_{CDA\cdot DHZ}| - |F_{CDA\cdot ZEB-H_2O}|, \phi_{CDA\cdot ZEB}\}$ difference Fourier maps which are less sensitive to resolution-dependent series termination effects than are the refined coordinates and which therefore provide much more reliable indications of structural differences.

## RESULTS

We have observed several important differences between the two complexes. Since these differences are of modest magnitude, relative to the resolution of the X-ray amplitude data and to the estimated error in the coordinates, it is important to emphasize that our quantitative conclusions are strongly supported qualitatively by unambiguous difference Fourier maps (Figure 2).

After the first cycle of X-PLOR refinement, in which zinc and zinc ligands were omitted, the $\{|F_o| - |F_c|, \phi_c\}$ difference Fourier maps for both complexes showed a strong electron density peak with an orientation and shape similar to that observed in the transition-state analog complex, CDA·FZEB−$H_2O$. The corresponding inhibitors were built into the respective densities and refined. The refined structure of CDA·ZEB−$H_2O$ shows that the distance between C4 and the hydroxyl group O4 is $1.6_6$ Å. This distance is somewhat longer than the 1.43 Å specified by the energy parameters for the refinement restraints, suggesting that this bond is unusually long in the transition-state analog complex.

*A Trapped, Active-Site Water Molecule in the DHZ Complex.* The difference map from the first refinement cycle for the DHZ complex revealed an electron density peak between the inhibitor C4 atom and the zinc atom (Figure 2a), which was interpreted to be a water molecule and refined as an oxygen atom. The peak height of $8\sigma$ provides the evidence that a water molecule is trapped in the active site when DHZ binds. It refines with an occupancy of 1.0 and a *B*-factor of 13 Å$^2$; it is therefore well ordered.

The presence of the trapped water molecule in the CDA·DHZ complex suggests a close analogy between this complex and the Michaelis complex for the catalyzed deamination reaction. There is precedent for trapping a water molecule by adenosine deaminase bound to deazaadenosine, a similar, but distinct substrate analog inhibitor for adenosine deaminase (Wilson & Quiocho, 1993), which has an appropriate leaving amino group but in which the hydrolytic reaction is prevented by substitution of the adjacent imide nitrogen by a methylene group. DHZ itself can also be considered as a stable substrate analog inhibitor in the sense that attack by water at C4 across the N3−C4 bond is impossible, owing to the fourth hydrogen substituent at C4. Moreover, the trapped water molecule in the active site can be considered analogous to the substrate water that hydrates cytidine and the inhibitor, ZEB. The hydrate ZEB−$H_2O$ could, in principle, be generated in free solution by attack of water on ZEB and then bound. However, the hydrate constitutes only about 1 part in $10^7$ of the total pyrimidine ribonucleoside in solution (Frick et al., 1989). If inhibition were dependent on such infrequent encounters, the combination of the rare ZEB−$H_2O$ species with the enzyme would be expected to result in slow onset of inhibition (Kati & Wolfenden, 1989b). Since no delay of inhibition is observed (Frick et al., 1989), the covalent hydrate ZEB−$H_2O$ must be produced inside the active site. In the crystal structure of CDA·DHZ, the trapped water molecule appears to be positioned for attack on C4, and it is the only water in the active site. Therefore, it probably resembles the substrate water.

*Any Conformational Changes That Occur in the Enzyme Itself on Binding the Different Ligands Must Be Very Small.* The refined crystal structure of the transition-state complex

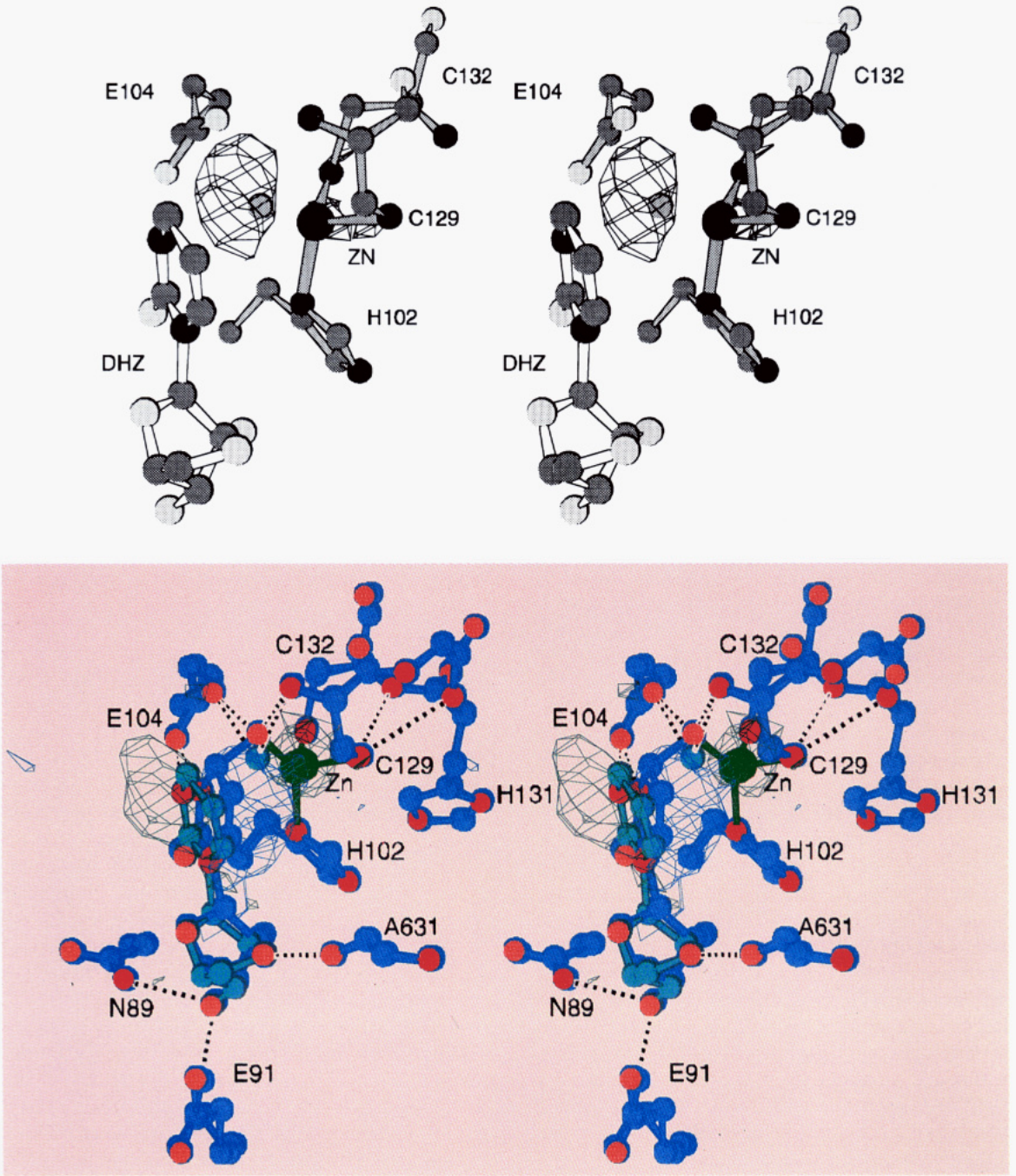


FIGURE 2: Difference Fourier maps showing the structural differences between the CDA·DHZ and CDA·ZEB complexes. (a. top) The $\{|F_o| - |F_c|, \phi_c\}$ difference map at the active site shows an electron density peak, contoured at $4\sigma$, for the water molecule trapped between C4 of DHZ and the zinc atom. (b, bottom) Superposition of the substrate-like inhibitor, DHZ, and the transition-state analog, ZEB, on the difference Fourier map calculated using coefficients $\{|F_{CDA\cdot DHZ}| - |F_{CDA\cdot ZEB}|, \phi_{CDA\cdot ZEB}\}$ and contoured at $3.5\sigma$. Positive contours to the left of the pyrimidine rings and surrounding the Zn−S$\gamma$132 bond are in green, as is the DHZ ligand. Negative contours between the pyrimidine ring and the zinc, the ZEB−$H_2O$ ligand, and the remaining groups in the active site are in blue.

CDA·ZEB−$H_2O$ is essentially identical to that of the CDA·FZEB−$H_2O$ transition-state analog complex (Betts et al., 1994). rms deviations between CDA·ZEB−$H_2O$ and CDA·FZEB−$H_2O$ are 0.15 Å for backbone atoms and 0.45 Å for side chains. The refined structure of CDA·DHZ is also identical to that of CDA·ZEB−$H_2O$ to within the same limits, except at the active site. rms deviations are 0.16 Å for backbone atoms and 0.41 Å for side chains. These comparisons provide an independent, and lower, estimate for coordinate error than that obtained from the statistical treatment of Luzzati (1952). In keeping with this observation, no significant variation was observed at specific residues, and no significant peaks or holes were found except at the active site in the $\{|F_{CDA\cdot DHZ}| - |F_{CDA\cdot ZEB}|, \phi_{CDA\cdot ZEB}\}$ difference Fourier map. In summary, no conformational change in the enzyme was observed between the CDA·DHZ complex and the two transition-state analog complexes.

However, there is a displacement of the DHZ inhibitor, relative to ZEB−$H_2O$ and FZEB−$H_2O$, as shown in Figure 2b. The pyrimidine ring of DHZ refines to a position where C4 is further by about 1.0 Å from the zinc atom than it is in the two transition-state analog complexes. The motion pivots the pyrimidine ring about the N3 atom, thereby leaving the hydrogen bond between N3 and O$\epsilon$1 of Glu104 essentially unchanged and shortening the hydrogen bond between the backbone amide nitrogen atom of Ala103 and the 2-one

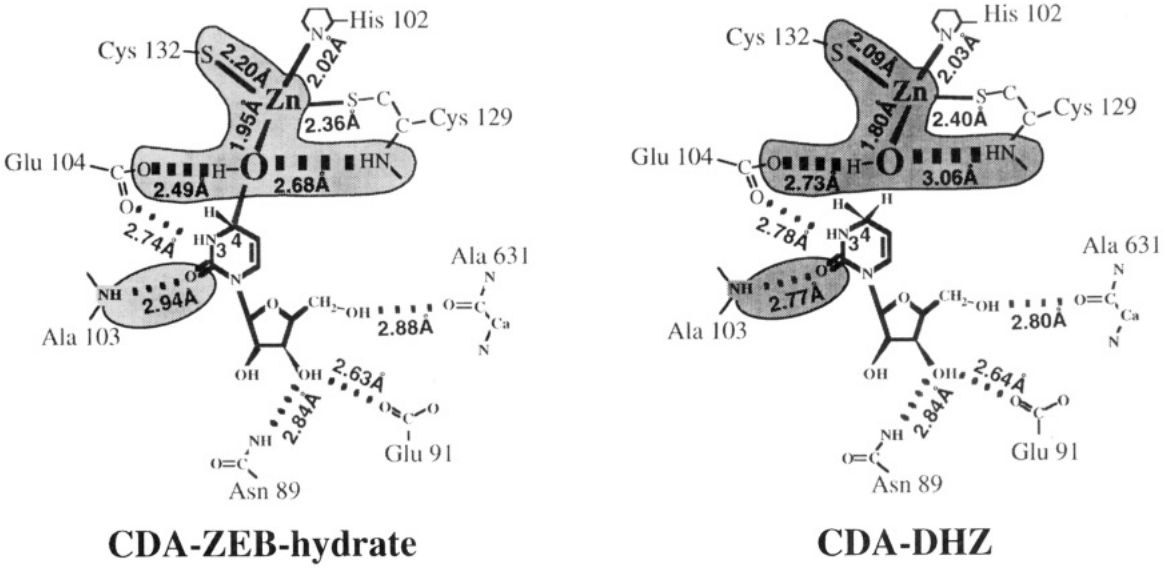


FIGURE 3: Localization of structural differences between CDA·ZEB and CDA·DHZ complexes to the neighborhood of the 4-hydroxyl group of the transition-state analog. Schematic diagrams of the inhibitor DHZ and the transition-state analog ZEB and their active site hydrogen-bonding interactions show that significant structural differences (shaded) are limited to the coordination of the zinc-bound water molecule and to the hydrogen bond to the 2-carbonyl oxygen. As elsewhere, residue numbers greater than 400 refer to interactions of the inhibitors with the other monomer across the dimer interface.

oxygen atom. These relative shifts in the refined structures are confirmed by the $\{|F_{CDA\cdot DHZ}| - |F_{CDA\cdot ZEB}|, \phi_{CDA\cdot ZEB}\}$ and $\{|F_{CDA\cdot DHZ}| - |F_{CDA\cdot FZEB}|, \phi_{CDA\cdot FZEB}\}$ difference Fourier maps using experimental amplitudes and calculated phases from the final models with the inhibitors omitted from the structure factor calculation.

*Most Binding Interactions Are Conserved in the Two Complexes.* In other respects, all three inhibitors, DHZ, ZEB−$H_2O$, and FZEB−$H_2O$, are bound by the enzyme in the same way (Figures 2b and 3). Interactions that are essentially the same in each complex include the hydrogen bond between O$\epsilon$2 of Glu104 and N3 on the pyrimidine ring and those between the ribose-3′- and 5′-hydroxyl groups and residues 89, 91, and 631 across the dimer interface. The ring displacement leaves these hydrogen-bonding interactions essentially unchanged, nor does it lead to bad steric conflicts. Since the enzyme structure itself is also unchanged by binding DHZ, the ring displacement would not be expected to introduce a significant binding energy difference between DHZ and the two transition-state analogs.

*Localized Structural Differences.* In contrast, the trapped water molecule in the DHZ complex makes different interactions from those observed for the 4-hydroxyl groups of the two transition-state analogs. Instead of making a covalent bond to C4 of the inhibitor DHZ, it lies about 2.5 Å from C4. As this distance is well within the restraining van der Waals close contact distance of 2.8 Å, it appears to constitute a bad steric conflict. The three remaining interactions observed for the hydrated ZEB inhibitors, including the covalent bond to zinc and hydrogen bonds with O$\epsilon$1 of Glu104 and the backbone amide nitrogen of Cys129, are altered in the DHZ complex. The zinc−oxygen distance is $0.1_5$ Å shorter, and the two hydrogen bond distances are longer in the DHZ complex by $0.2_4$ Å and $0.3_8$ Å, respectively, because the oxygen atom recedes from its position in the transition-state analog complex (Figure 2b).

The zinc coordination sphere also undergoes significant rearrangement. The positive difference density surrounding the zinc−S$\gamma$132 bond in the $\{|F_{CDA\cdot DHZ}| - |F_{CDA\cdot ZEB}|, \phi_{CDA\cdot ZEB}\}$ difference map (Figure 2b) implies a shortening of that bond in the DHZ complex. This shortening is confirmed by the refined coordinates, which suggest that the zinc−sulfur bond shortens by about 0.1Å and that both the zinc and S$\gamma$132 move away from the DHZ pyrimidine ring as the water molecule moves from its transition-state position. These correlated motions are facilitated by rotation about the $C_\alpha$−$C_\beta$ bond of Cys132 whose orientation is well suited to this purpose (Figure 2b). In fact, this motion is the only one that seems to be permitted by the environment of the three CDA zinc ligands. The position of S$\gamma$129 is restrained by two NH−S hydrogen bonds (Carter et al., 1974; Berg, 1988; Summers et al., 1990; Schwabe & Klug, 1994) from backbone amides of residues His131 and Cys132 and is therefore likely to be energetically costly to displace. The imidazole of His102 is similarly restrained by an unusual configuration, described previously (Betts et al., 1994), involving a hydrogen bond to the backbone carbonyl oxygen atom of Phe633 from the other monomer. The extent to which these changes represent accommodation to the presence of both the DHZ ligand and the trapped water molecule may be clarified by refinement of the ligand-free structure now underway. However, the altered Zn−S$\gamma$132 and Zn−O distances probably do reflect electronic alterations in zinc ligation related to changes in how the trapped, ground-state water and 4-hydroxyl group of the transition-state analogs interact with the enzyme and pyrimidine ligand.

## DISCUSSION

The $K_i$ values for DHZ and for the 3,4-hydrate (ZEB−$H_2O$) (Frick et al., 1989; Wolfenden & Kati, 1991) differ by a factor of $2 \times 10^7$, implying a difference of 10.1 kcal/mol in their free energy of binding. This difference might be expected to arise from two possible sources. First, the respective ligands, ZEB−$H_2O$ versus DHZ plus water, may face different desolvation energies as they leave solution for the enzyme active site. Second, the desolvated ligands may acquire energetically different interactions with the enzyme once they are bound. Because of the high degree of

similarity between the two pyrimidine ligands, desolvation energy differences can be estimated reliably on the basis of model compounds. Structural differences obtained from this work, shown in Figures 2 and 3, place stringent limits on the possible significant contributors to the differential binding affinity from enzyme−ligand forces. As illustrated in Figure 1b, these can be further broken down into direct interactions between enzyme and ligand(s), changes in the ligand's conformation, and changes in the enzyme's conformation.

*Contributors to the Differential Binding Affinity.* Free energies of desolvation undoubtedly differ for DHZ and ZEB−$H_2O$, affecting their relative binding affinities. Free energies of solvation of organic molecules have been found to be a nearly additive function of their constituent groups (Hine & Mookerjee, 1975). Since our crystal structures indicate that the active sites are both inaccessible to solvent water, the free energies of desolvation of the critical regions where the structures of DHZ and ZEB−$H_2O$ differ can be estimated by comparing the vapor pressures of secondary alcohols with those of alkanes, over their dilute aqueous solutions. Alcohols exceed alkanes in hydrophilic character by a factor of approximately $10^5$ (Butler, 1937; McAuliffe, 1966) or 6.8 kcal/mol, so that desolvation of DHZ is expected to occur more easily than that of ZEB−$H_2O$ by a similar factor. However, the present experiment shows that desolvation of the trapped water molecule in the DHZ complex must also be taken into account. That contribution can be obtained from the vapor pressure of water (Washburn, 1928) and is 3.9 kcal/mol. Accordingly, differential desolvation of the two inhibitors is likely to make a significant contribution to the differential binding energy, actually favoring the binding of DHZ by 2.9 kcal/mol. Thus, the inherent affinities of the desolvated ligands differ not by 10.1 kcal/mol but rather by 13 kcal/mol.

We have demonstrated in the present study that the inherent differential binding affinity for the desolvated ligands arises from highly localized differences in structure, associated almost exclusively with the 4-hydroxyl group of ZEB−$H_2O$ (Figure 3). The DHZ and ZEB−$H_2O$ ligands both bind to CDA with the same *anti* conformation of the *N*-glycosidic bond and with a C-3′ *endo* sugar pucker. Moreover, the two crystal structures reveal no detectable conformation change in the enzyme. Interactions of the ribose moiety with the enzyme are the same for both DHZ and ZEB−$H_2O$ in the sense that the carbon frameworks are nearly superimposable (Figure 2b) and that they form similar hydrogen bonds with the enzyme (Figure 3). The hydrogen bond between O$\epsilon$2 of Glu104 and N3 of the pyrimidine ring is essentially unchanged in the two complexes. Thus, the energetics of interactions between CDA and these parts of the two ligands are unlikely to be significantly different.

The displacement of DHZ away from the trapped water with respect to ZEB−$H_2O$ and the concerted motions of the zinc, water, and S$\gamma$ of Cys132 in the opposite direction (Figure 2b) are difficult to evaluate energetically but could represent strain in either or both complexes, relative to the uncomplexed enzyme. The zinc−water and zinc−S$\gamma$ distances are both shorter in the DHZ complex, and there is a surprisingly close contact of 2.5 Å between DHZ and the trapped water molecule. The energetic contribution of that interaction is hard to estimate because the repulsive energy potentials change inversely with the 12th power of the interatomic distance, $1/d^{12}$, and are therefore highly sensitive to coordinate errors. The accuracy of the coordinates therefore leaves considerable latitude in estimating the strain energy. By two different estimates,[2] the distance of 2.5 Å corresponds to a repulsive energy of ~10−12 kcal/mol. Although this value is high, the slope of the functional dependence is steep, and an interatomic distance of 2.6 Å provides an estimate of ~6 kcal/mol. These observations all suggest that there is not enough room in the active site to accommodate both DHZ and the trapped water molecule without unfavorable van der Waals contacts between them and that our structures imply reasonable energetic contributions to differential binding energy. Hence we believe that strain is present in the DHZ complex, contributing to the relative weakness of DHZ binding. That strain may be partially compensated, however, by the fact that the hydrogen bond between the backbone NH of Ala103 and the 2-keto oxygen atom of the ring appears slightly shorter in the DHZ complex.

The 4-hydroxyl group of ZEB−$H_2O$ itself makes stronger interactions with the enzyme, compared with those of the water molecule in the DHZ complex. The hydrogen bonds to the ZEB hydroxyl group from O$\epsilon$1 of Glu104 and from the backbone amide nitrogen atom of Cys129 are substantially shorter, by $0.2_4$ and $0.3_8$ Å, respectively, than those made by the trapped water to the same groups in the DHZ complex (Figure 3). The average oxygen−oxygen distance between the hydroxyl and Glu104 in the two transition-state analog complexes is 2.45 Å, whereas all other hydrogen bond distances in the active site exceed 2.6 Å, raising the possibility that it becomes a strong, "low-barrier" hydrogen bond (Kreevoy & Liang, 1980; Gerlt & Gassman, 1993; Cleland & Kreevoy, 1994; Loh & Markley, 1994) in the ZEB−$H_2O$ complex and, by implication, in the transition state. Development of covalent character in the C4−O4 bond converts the zinc-bound oxygen into a species more closely resembling an alkoxide group than a hydroxyl group. The consequent weakening of the bond between O4 and its proton would be expected to depress the p$K_a$ of this hydroxyl group proton toward that of the carboxylate and permit these two groups to interact more strongly in the transition-state complex.

This possibility also appears to be consistent with the apparent change in the zinc coordination sphere, particularly the lengthening of the Zn−S$\gamma$132 bond in the transition-state analog complexes. A developing negative charge on the O4 atom would undoubtedly also weaken the interaction between it and the zinc atom and between the zinc atom and its remaining ligands, perhaps leading to a reconfiguration of the coordination sphere and the lengthening of the Zn−S$\gamma$132 bond. The geometry of the Cys132 thiolate ligand appears ideally poised to contribute to the catalytic mechanism by "buffering" the bond valence sum on the zinc atom (Thorp, 1992; Liu & Thorp, 1993).

Thus, there appear to be two possible sources for the 13 kcal/mol of intrinsic differential binding affinity between the desolvated ligands: steric strain and strengthened interactions with the hydroxyl group itself. Strain is evident in the complex with DHZ, arising from unfavorably close interac-

[2] The 6-12 potential implemented in the program SIGMA (Hermans, 1993) was used as follows: $\Delta G$ (kcal/mol) = $-(40 \times 51)/(4.182 \times d^6) + (1960 \times 1623)/(4.182 \times d^{12})$. Alternately, the van der Waals energy was estimated using X-PLOR, giving comparable values.

tions with the trapped water molecule, which cannot hydrate DHZ. These effects are expected to diminish the affinity of CDA for DHZ and seem capable of contributing several kilocalories per mole to the differential binding affinity. Since we can rule out any conformational changes in enzyme or ligand as possible sources for the difference in binding energy, we conclude that strengthened interactions with the hydroxyl group must also play a major role. Two hydrogen bonds with the 4-hydroxyl group are strengthened in the ZEB−$H_2O$ complex. One of the hydrogen bonds, to Glu104, involves a negatively charged partner and a bonding distance significantly shorter than that observed for normal hydrogen bonds. Strengthening of that bond upon forming ZEB−$H_2O$ appears to be corroborated by the increased zinc−S$\gamma$132 bond distance; it undoubtedly represents a considerable contribution to the differential binding affinity, probably exceeding the value of ~4 kcal/mol estimated by directed mutagenesis experiments (Fersht et al., 1985). If it is indeed a low-barrier hydrogen bond, then it could provide substantially more stabilization energy in the ZEB−$H_2O$ and transition-state complexes.

Mutagenesis studies (Carlow et al., 1994) indicated that removing the Glu104 side chain reduces enzymatic activity by more than 6 orders of magnitude, consistent with a $\Delta$-($\Delta G$) value of 8 kcal/mol for $k_{cat}/K_m$ (Wolfenden & Kati, 1991). Although Glu104 probably contributes other important catalytic functions (Betts et al., 1994), this hydrogen bond is certainly critical for catalysis. It is also likely that alteration of the zinc coordination sphere, implied by the lengthening of the Zn−S$\gamma$132 bond, also contributes to the unusual stability of the transition-state complex by facilitating the development of increased negative charge on the O4 atom. These conclusions provide a particularly clear picture of how changes in enzyme−ligand interactions may lead to catalysis.

*The "Entropy Trap" Mechanism.* The trapped water molecule and the interactions between the enzyme and DHZ also illustrate the "entropy trap" mechanism previously proposed for ADA (Kati & Wolfenden, 1989a,b). The structure of CDA·DHZ indicates that the substrate analog DHZ and the water molecule are bound to the enzyme and aligned properly for water addition, as is also presumably the case when the substrate cytidine approaches the active site before the transition state. An entropy loss would be expected to accompany this process. For nebularine in dilute solution, a small enthalpy change accompanies the nonenzymatic covalent addition of water (−1.2 kcal/mol), but the entropic contribution to this process, $T\Delta S$, is highly unfavorable, approximately −10.5 kcal/mol at 25 °C (Kati & Wolfenden, 1989a). The net free energy change for nebularine hydration, +9.3 kcal/mol, falls slightly short of the differential binding affinity between the two ligands described here. As in the case of ADA, CDA can be considered to compensate the loss of entropy by developing strong binding energies in the covalently hydrated transition state.

*Interpreting Substituent Effects.* Our analysis of the ligand substituent effect shown in Figure 1 illustrates a potential pitfall of using discrete chemical modifications (based on structural alteration of a ligand or on mutation of a binding site) to estimate the contributions from a single chemical group to binding affinity. Specifically, it should be noted that the van der Waals repulsion between DHZ and the trapped water molecule would lead to a relatively *tighter* binding of DHZ in the absence of the trapped water molecule. The water molecule itself must thus retain a considerable amount of the binding energy, stabilizing the hydroxyl group in the transition-state analog complex. The presence of the trapped water molecule therefore shows that it would be an oversimplification to suppose that comparison of binding affinities provides a direct indication of differences in the aggregate strengths of bonds joining the active site with specific functional groups in matched compounds such as ZEB−$H_2O$ and DHZ. In the context of the present findings, it is of interest to identify the assumptions underlying this supposition and to examine the likely effect of the failure of these assumptions on the magnitude of the binding contribution ascribed to the missing group, in this case the hydroxyl group.

First, the shape of the active site is assumed to be the same in the enzyme's complex with each of the two ligands. In most real cases in which interactions between an enzyme and inhibitor are strong, the shape of the binding site probably differs to some extent for the differing ligands, as suggested in Figure 1 and as is observed in the present case. To the extent that the enzyme adjusts its shape to accommodate different ligands, these adjustments would be expected to level the difference between the observed binding affinities, reducing its value compared with the value that would be observed if the enzyme's structure were identical in the two complexes.

Second, when a substituent is deleted from either an enzyme or a ligand, a water molecule may take the place of the missing group, as is also observed in the present case. At equilibrium, the enzyme's water-containing complex with DHZ is evidently more stable than any hypothetical DHZ complex from which that group is absent, *despite the unfavorable van der Waals interaction.* However, it is the latter complex whose stability one would wish to determine in order to evaluate the binding contribution of the missing hydroxyl group. It follows that the equilibrium constant for converting the CDA·ZEB−$H_2O$ complex into a CDA·DHZ complex without entry of a water molecule must be less than $10^{-7}$, i.e., the hydroxyl group's contribution to the negative free energy of binding amounts to more than the 13 kcal indicated by the ratio of dissociation constants, corrected for desolvation effects.

This conclusion has significant implications for the interpretation of difference energies obtained by chemical modification and/or mutagenesis. Such analyses are widespread because of their potential utility in efforts to design better enzyme inhibitors and to engineer new catalysts. Leveling effects of the kinds suggested above can lead to errors in evaluating the strengths of bonds formed in enzyme−ligand complexes from observed substituent effects, here replacement of the 4-hydroxyl group of ZEB−$H_2O$ by a hydrogen atom (Figure 1). The presence or absence of these leveling effects can only be established by examination of the structures as we have done here. The contribution of a substituent to binding affinity will be underestimated when such leveling effects are ignored.

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