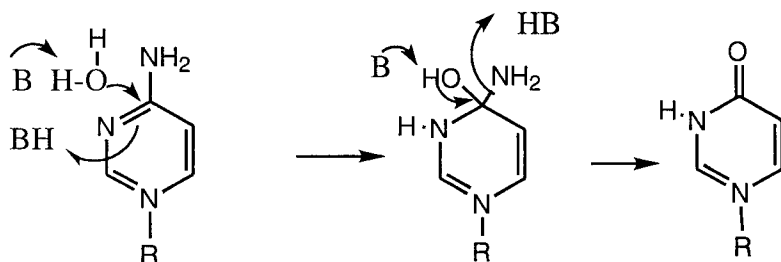
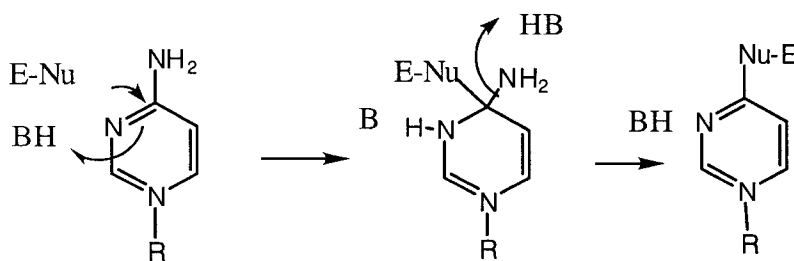


1. There are several mechanisms that could be considered based on your knowledge of serine, cysteine, aspartate and metallo-proteases. Without seeing any structures any one, or a combination of several mechanisms, are possible. The simplest mechanism would involve general base catalysis (GBC), that is, conversion of water to a better nucleophile for attack at C-4 of the pyrimidine to form a tetrahedral intermediate. It is likely that the N-3 nitrogen of the pyrimidine would be protonated by a general acid catalyst (GAC). The N3 position of cytosine has a pKa (4.5) in the physiological range. You will probably not know this number, but it is always important when looking at a new reaction to become familiar with the properties of your starting material and products. The tetrahedral intermediate generated would then collapse to form product. A GBC, would deprotonate the OH at C-4 and a GAC would facilitate leaving of ammonia. It is in general true that nature has found ways to minimize the number of general acid and base catalysts and thus can reuse an amino acid side chain for more than one function as you have seen with the histidine in chymotrypsin. Using an aspartate protease type mechanism D or E could function as B and or BH and switch roles.



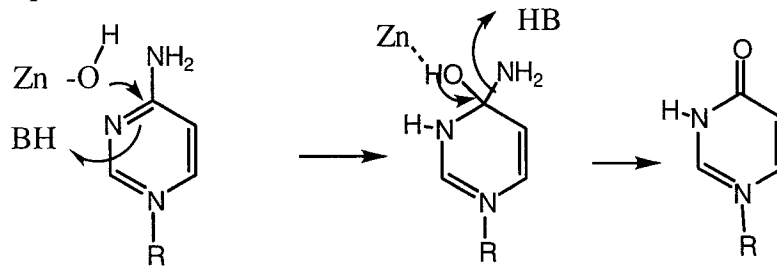
One could also easily write a mechanism with a covalent intermediate to the side chain of an amino acid residue (C or S as in the case of cysteine and serine proteases, respectively). The



Covalent intermediate where Nu is a nucleophile. In the second step you hydrolyze the intermediate

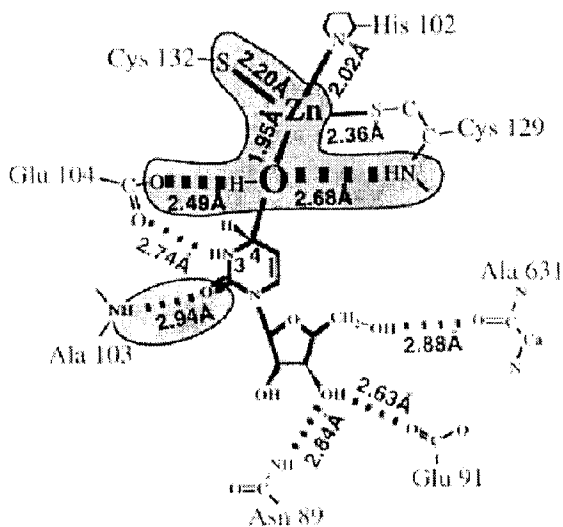
mechanism would be similar to that above except that you first form a td intermediate with the enzyme, followed by loss of ammonia. This intermediate then needs to be hydrolyzed to form

product. Alternatively using a Zn^{2+} protease as a prototype, the zinc could function to make water more nucleophilic and/or stabilize the tetrahedral (td) transition state or intermediate. From

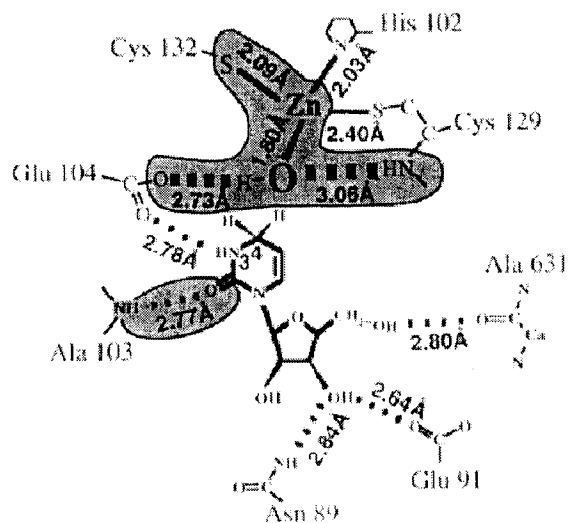


the mechanisms without covalent enzyme intermediates, ZEB-H₂O and DHZ could be transition state analogs of the td intermediate. It is harder to envision how these inhibitors would bind if a covalent enzyme intermediate were actually involved in catalysis. Since all of the binding energy available from the inhibitor is used for binding and not catalysis, ZEB-H₂O and DHZ, would be expected to bind tightly. Recall from class and the Wolfenden calculations that $k_{cat}/K_m/k_{uncat} = 1/K_{TS}$. Looking at the putative td intermediate, ZEB-H₂O is a better model for this ts than DHZ. As you will see subsequently, there is an additional reason why ZEB-H₂O binds so much more tightly than DHZ. Remember enzymes are thought to be complementary to the transition states of the reactions that they catalyze. ZEB on the other hand looks like a ground state analog either the substrate or product.

2. The X-ray structure of CDA was solved in 1994 and the structures of ZEB-H₂O and DHZ were published in *Biochemistry* 34, 4516 (1995). The initial structure in 1994 revealed a zinc in the active site. This observation was a complete surprise. The figures below are taken directly from the paper, as are the reported distances. One can tell from the structures that Zn^{2+} is coordinated in a tetrahedral fashion to C129, C132 and H102 and importantly to the 4-HO of ZEB-H₂O. When one examines the distances, of particular interest is the short distance between the E104 oxygen and the oxygen of the 4-OH of the inhibitor (2.49 Å). This distance is less than the sum of the van der waals radii and suggests the presence of a LBHB (low barrier hydrogen bond). In fact the observation of this putative H bond and its potential importance in catalysis via transition state stabilization, led to the preparation of E104A mutant. In addition the backbone amide NH of C129 appears to form a H bond with the same 4-HO of the inhibitor. You have previously seen the importance of amide backbone NHs in the OMP DC case. Of particular interest is a comparison of the structures of ZEB-H₂O and DHZ. DHZ has two hydrogens at C-4. Initially, without the structure of DHZ bound to CDA, one might have drawn strong conclusions about the importance of a HO in the observed rate acceleration. However, when one looks at the structure of DHZ with CDA one observes a water bound to the zinc!! The presence of water in this case can only be identified by crystallographic methods. Zinc is spectroscopically invisible due to its d10 configuration.

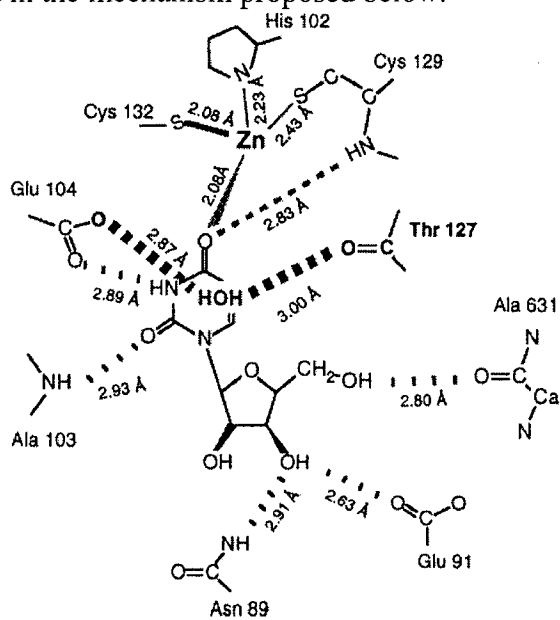


CDA-ZEB-hydrate

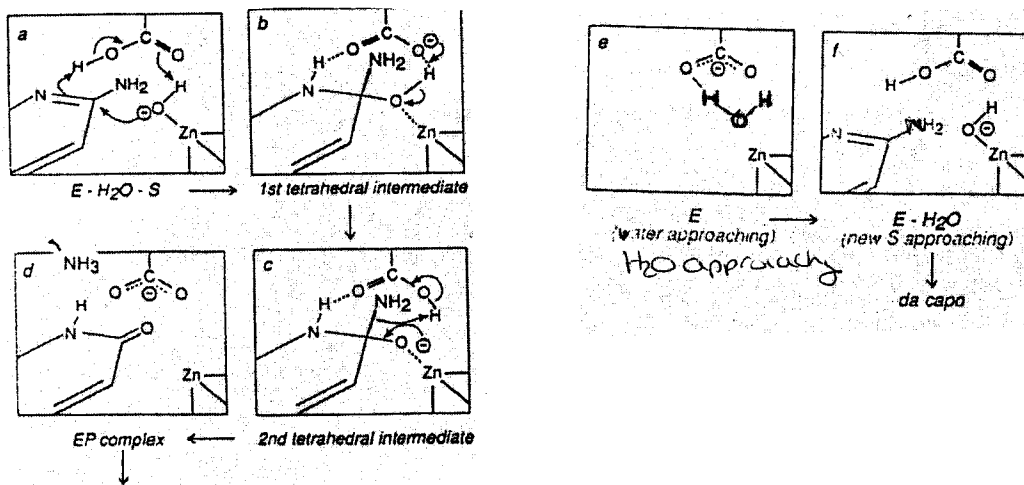


CDA-DHZ

The structure of uridine bound to CDA was reported in Biochemistry 36, 4768 (1997). The conclusions drawn from this structure are much more subtle and would require a great effort on your part or reading the authors conclusions and then looking to see if you believed these conclusions. One interesting distance is the zinc to the C4 carbonyl of uridine (2.08 Å, compared to the ts analog ZEB-H₂O above). A second interesting distance is the E104 relative to a water in the active site. The structure with ZEB-H₂O indicates that this E is intimately involved in catalysis. Now if you have the heteratoms on, you can see water molecules. The authors claim there is a very interesting water that interacts with E104 (2.87 Å, H bond distance). They claim the water is in the position of the departing ammonia and thus that E104 may now be functioning to protonate the leaving group as indicated in the mechanism proposed below.



3. The mechanism proposed by Wolfenden based on these structures is shown below. The model is that the zinc has activated the water for nucleophilic attack on C-4 of cytidine and that N3 of cytidine is protonated by E104 which is in the protonated state (pKa must be perturbed for this residue to be protonated and this point would need to be tested experimentally, the basis for this protonation is described subsequently). They propose the td intermediate structure shown below. Now they propose that E104 can protonate the ammonia leaving group concomitant with deprotonation of the C4-OH of the td intermediate. The model further suggests that the protonated E104, in the first step, results from deprotonation of the water bound to the zinc, making it ready for reaction with the substrate. The E104 and the zinc thus play essential roles in catalysis.



4. a. The rationale would be that the enzyme binds the ts more tightly than the gs so that you are not in a thermodynamic hole. In the case of ZEB-H₂O all of the binding energy can be used to bind. There is no reaction. Thus the putative LBHB and the tighter coordination to the zinc could be related to the observed factor of 10⁷. Remember 1.38 kcal/mol, unique to the ts state, can give you a factor of 10 in rate acceleration.
- b. The difference is the hydroxyl at C4. This hydroxyl interacts intimately with the E104 and with zinc. In the case of DHZ, described above, without a structure you would conclude that the HO plays a very important role. The structure reveals that a water is now at the active site bound to zinc and the binding is still weaker. This is because you do not have enough room to fit everything into the active site to maximize interactions.
- c. Each factor of 10 requires 1.38 kcal/mol at 25 C. Use the Eq I gave in class

$$k_1/k_2 = e^{-\Delta\Delta G/RT}$$

The value is approximately 13 kcal.

5. As discussed in class the relative contributions to rate acceleration of binding energy, GA, GBC, etc are difficult to assess. E104 in the proposed mechanism can act as both GB and GA

catalyst. In addition the zinc also functions to activate the water for nucleophilic attack and to stabilize the ts. The best we can do at present is to think about the structure of the putative ground state (uridine) and the putative transition state (ZEB-H₂O) realizing that this is a static picture in each case. One must further realize that we cannot synthesize an actual ts analog (highest point on the reaction coordinate diagram, short life time) and that the gs structure may reflect the predominant conformation of an ensemble of structures. The dominant structure may not be the one that leads to catalysis. One can see by looking at the structures, that stabilization of the ts is a possibility. We see a putative LBHB between glutamate and the ts analog and we see a shorter distance between the intermediate and zinc than between the uridine and zinc. Wolfenden claims from the structure of uridine that the gs is also destabilized. This observation requires extensive analysis of the structures and bond angles. Thus, as with almost all enzymes, the rate acceleration observed is a composite of mechanisms. It is difficult to dissect out individual contributions.

6. The structure seems to suggest that zinc can function to make water more activated toward nucleophilic attack on the pyrimidine. The transition state is stabilized by several, perhaps unique H bonds and by coordination to the zinc. If you look at the structure of CDA that has been incubated with ZEB, you will see that the enzyme has catalyzed the addition of water to C-4 of the substrate. Since there is no leaving group present, the normal substrate has been converted to a potent inhibitor by the enzyme. ZEB now possesses a HO that can coordinate to the zinc. The change in absorbance is due to the loss of conjugation upon addition of water.

