Testing the Limits of Protein–Ligand Binding Discrimination with Transition-State Analogue Inhibitors

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Received October 10, 1990 (Revised Manuscript Received May 7, 1991)

The functions of many globular proteins depend on a remarkable sensitivity to subtle differences in structure between other molecules. These powers of binding discrimination, familiar in the biological properties of hormone receptors and antibodies, reach an acme of expression in the active sites of enzymes, where they appear to be directly responsible for the catalytic process. The theory of absolute reaction rates implies that the function of any catalyst depends on its special affinity for the altered substrate (S‡) in the transition state, binding that species more tightly than the substrate in the ground state (S) and diminishing the difference in energy that limits the rate of the uncatalyzed reaction.[1] These two forms of the substrate, with many structural features in common, differ in their binding affinities for an enzyme's active site in water by a factor that equals or exceeds the rate enhancement that an enzyme produces in water.[2] That factor has always been known to be large, but its potential magnitude has come to be appreciated only recently. Enzyme rate enhancements are commonly in excess of 10^{12}, and some enzymes[3] are believed to enhance reaction rates by factors as large as 10^{17}.

It would be useful to know whether these powers of binding discrimination are based on recognition by the enzyme of numerous small differences in structure between S‡ and S, or whenever they arise mainly from a few binding interactions that are present in ES‡ but absent from ES. An answer to that question would have a significant bearing on the design of enzyme inhibitors and on efforts to modify the activities of existing enzymes. It should be possible, by comparing the binding affinity of an enzyme for S‡ (or a stable analogue of S‡) with the enzyme's binding affinity for an otherwise similar compound, lacking a particular substituent but identical in other respects, to analyze the distinctive binding affinity of S‡ in terms of the contribution made by that single substituent. This Account describes some apparent contributions made by ligand hydroxyl groups to enzyme affinities for transition states and their analogues, analyzed in this way. The results suggest that, for some enzymes, a single ligand hydroxyl group can furnish much of the rate enhancement that the enzyme produces.

Effects of Single Substituents on Binding Discrimination Inferred from Enzyme Reaction Rates on Different Substrates

How large are the effects of substituents on the rates of reactions of substrates in enzyme-catalyzed reactions? For convenience, our discussion will be confined to substrate hydroxyl substituents, which are especially abundant in molecules of biological importance and serve as one obvious feature by which substrates might be recognized by proteins. Ribose kinase[4] and glycerol dehydrogenase,[5] for example, appear to be indifferent to certain hydroxyl groups in substrates, whose reactivities are hardly affected by their presence or absence. In such cases, it seems natural to suppose that the hydroxyl group in question remains exposed to the solvent throughout the course of the reaction. Hydroxyl groups have large negative free energies of solvation (see below) for which the enzyme would otherwise need to compensate exactly, in order to produce the null effect observed when the reactivities of hydroxyl-containing substrates are compared with those of hydrogen-substituted substrates.

In contrast with the examples mentioned above, other enzymes are capable of sharp discrimination between substrates in which hydroxyl groups are present or absent. For β-glucosidase (IV), β-galactosidase (III), a catalytic RNA molecule (V), and tyrosyl-tRNA synthetase (VI), values of k_{cat}/K_m (the second-order rate constant for enzyme–substrate reaction) indicate that the normal, hydroxyl-containing substrate is preferred over the corresponding hydrogen-containing substrate by factors as large as 10^5 (see entries in Table I and Figure 1). The term $[k_{cat}/(K_m k_{non})]$, where k_{non} is the rate constant for reaction in neutral solution in the absence of enzyme, is roughly equivalent to the binding affinity of the altered substrate in the transition state.[6] If the rates of the nonenzymatic reactions are similar for the hydroxyl- and hydrogen-containing compounds, then the values for these enzymes in Table I represent the apparent contribution of a hydroxyl group to transition-state stabilization. For β-glucosidase and

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(1) Polanyl, M. *Z. Elektrochem.* **1921**, *27*, 143. Because the energy of the adsorbed substrate is lower than that of the substrate in solution, adsorption of the substrate does not constitute chemical activation. Instead, the forces of adsorption work in such a way that the adsorbed substrate (although not itself activated) is easily activated, so that the equilibrium for forming the transition state in its chemical transformation is more favorable on the catalyst than in free solution.

(2) For a recent review, see Wolfenden, R.; Frick, L. In *Enzyme Mechanisms*; Page, M. I., Williams, A., Eds.; Royal Society of Chemistry: London, 1987; pp 97–122.

(3) Guthrie, J. P. *J. Am. Chem. Soc.* **1977**, *99*, 3391.

(4) Agranoff, B. W.; Brady, R. O. *J. Biol. Chem.* **1956**, *219*, 221.

(5) Burton, R. M. *Methods Enzymol.* **1955**, *1*, 397.

(6) Wolfenden, R. *Mol. Cell. Biochem.* **1974**, *3*, 207.

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Table I.
Apparent Contributions of Single Hydroxyl Groups to Binding of Hydroxyl- vs Hydrogen-Containing Ligands

enzyme[a]	hydroxyl ligand/hydrogen ligand	$\Delta\Delta G$
I. cytidine deaminase[b]	3,4-H_2uridine/4-deoxy-3,4-H_2uridine	−10.1
II. adenosine deaminase[c]	6-OH-1,6-H_2PuR/1,6-H_2PuR	−9.8
III. β-galactosidase[d]	galactosides/2-deoxygalactosides	−7.6
IV. β-glucosidase[e]	glucosides/2-deoxyglucosides	−7.3
V. tetrahymena ribozyme[f]	G_2CCCUCUA$_5$/dCCCUCUA$_5$	−7.4
VI. Tyr-tRNA synthetase[g]	tyrosine/phenylalanine	−7.0
VII. glycogen phosphorylase[h]	glucose-1-P/3-deoxyglucose-1-P	−6.3
VIII. glycogen phosphorylase[g]	glucose-1-P/6-deoxyglucose-1-P	−5.9
IX. fumarase[i]	malate/succinate	−5.7
X. adenosine kinase[j]	adenosine/2′-deoxyadenosine	−5.6
XI. DOPA decarboxylase[k]	DOPA/tyrosine	−5.1
XII. leucine aminopeptidase	leucinal hydrate/leucinol[l]	−5.1
XIII. leucine aminopeptidase	amastatin/deoxyamastatin[m]	−4.7

[a] Roman numerals refer to structures in Figure 1. [b] K_i(H)/K_i(OH). Frick et al.[14] [c] K_i(H)/K_i(OH). Kati and Wolfenden.[16] [d] [k_{cat}/k_m(H)]/[k_{cat}/k_m(OH)]. Sinnott, M. L.; Souchard, I. J. *Biochem. J.* **1973**, *133*, 89. Results compared with the following: Wentworth, D.; Wolfenden, R. *Biochemistry* **1974**, *13*, 4715. [e] [k_{cat}/k_m(H)]/[k_{cat}/k_m(OH)]. Roeser and Legler.[7] [f] [k_{cat}/k_m(H)]/[k_{cat}/k_m(OH)]. Herschlag, D.; Cech, T. R. *Nature* **1990**, *344*, 405. Provisional value, since ligands differ by more than one OH group. [g] [k_{cat}/k_m(H)]/[k_{cat}/k_m(OH)]. Fersht, A. R.; Schindler, J. S.; Tsui, W.-C. *Biochemistry* **1980**, *19*, 5520. [h] [k_{cat}/k_m(H)]/[k_{cat}/k_m(OH)]. Street, I. P.; Rupitz, K.; Withers, S. G. *Biochemistry* **1989**, *28*, 1581. [i] K_i(H)/K_m(OH). Massey, V. *Biochem. J.* **1953**, *55*, 172. [j] K_i(H)/K_m(OH). Lindberg, B.; Klenow, H.; Hansen, K. *J. Biol. Chem.* **1967**, *242*, 350. [k] K_i(H)/K_m(OH). Lovenberg, W.; Weissbach, H.; Udenfriend, W. *J. Biol. Chem.* **1962**, *237*, 89. [l] K_i(H)/K_i(OH). Andersson, L.; Isley, T. C.; Wolfenden, R. *Biochemistry* **1982**, *21*, 4177. Results compared with the following: Frick, L.; Wolfenden, R. *Biochim. Biophys. Acta* **1985**, *829*, 311. [m] K_i(H)/K_i(OH). Rich, D. H.; Moon, B. J.; Harbeson, S. *J. Med. Chem.* **1984**, *27*, 417.

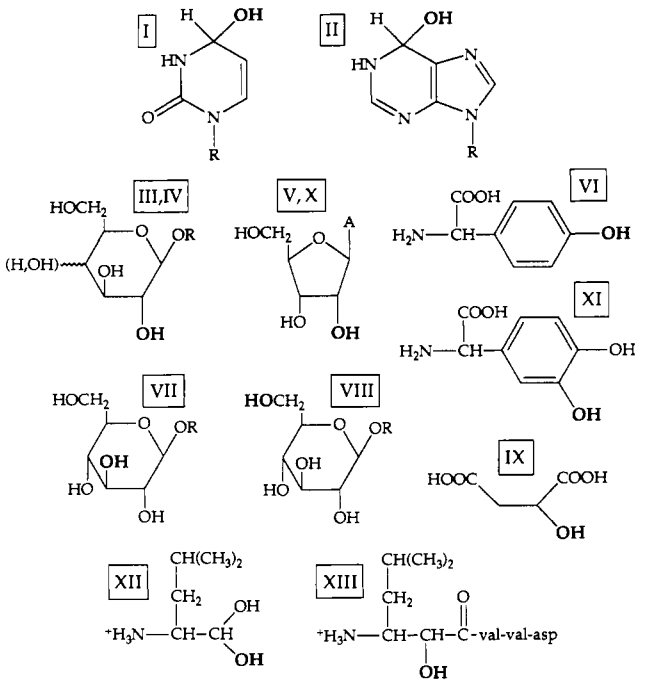

Figure 1. Hydroxyl groups, shown in boldface, that make large contributions to enzyme affinities for ligands in the transition state (Table I). Roman numerals refer to entries in Table I.

β-galactosidase, the nonenzymatic reaction is more rapid for the hydrogen-containing compound than it is for the 2-hydroxyl-containing compound, so that the contribution of the 2-hydroxyl group to transition-state binding by the enzyme is even greater than the rate comparison suggests.[7]

In interpreting these differences in reaction rate, it is useful to remember that the position on the reaction coordinate at which the hydroxyl-containing substrate reaches its transition state may differ from the position on the reaction coordinate at which the hydrogen-containing substrate reaches its transition state. Less probably, the actual mechanism of transformation of the two substrates may not be the same, so that the fundamental nature of the transition state is different for the hydrogen-containing substrate than it is for the hydroxyl-containing substrate. The structure of the enzyme's active site in the transition state might also change, by bond rotations, for example, in such a way as to accommodate differences between the structures of the substrate and provide better contact in the transition state. Figure 2 suggests that these effects, occurring singly or in combination, would tend to exert a leveling influence on the relative rates observed for the two reactions, leading to *underestimation* of the binding contribution made by the hydroxyl group that has been deleted from the substrate.[8]

Contributions of Single Hydroxyl Groups to Enzyme Affinities for Transition-State-Analogue Inhibitors

The transition state itself presents a "moving target", whose exact structure is likely to remain elusive for the foreseeable future. In seeking to learn the levels of binding discrimination of which enzymes may be capable, it would be better to use molecules of stable structure, whose complexes can be described in exact terms. Stable analogues of high-energy intermediates in substrate transformation, approaching the transition state in structure, provide a suitable tool for this purpose because their binding affinities can be measured at equilibrium. This allows the contributions of hy-

(7) Roeser, K. R.; Legler, G. *Biochim. Biophys. Acta* **1981**, *657*, 321.

(8) Similar considerations apply to the results obtained when the structure of the enzyme is altered by replacing an active-site serine, for example, by alanine. By this complementary approach, amino acid substitutions have been found to make apparent contributions to free energies of binding in the transition state, inferred from values of k_{cat}/K_m, that fall in the range between −0.5 and −1.5 kcal/mol in cases where the partners are uncharged, and in the range between −3 and −6 kcal/mol for those cases in which one of the partners bears an electrostatic charge (Fersht, A. R.; Wells, T. N. C.; Leatherbarrow, R. J. *Trends Biochem. Sci.* **1986**, *11*, 321). These values may represent lower limits, if mutation leads to effects corresponding to those illustrated in Figure 2, involving a change in mechanism or a change in the position on the reaction coordinate where the transition state is reached. It is also possible that, in some cases, the structure of the protein may relax in such a way as to compensate for interactions that have been altered by mutation. In other cases, the near additivity of effects observed in multiple mutants suggests that amino acid alterations do not lead to major changes in protein structure (Carter, P. J.; Winter, G.; Wilkinson, A. J.; Fersht, A. R. *Cell* **1984**, *38*, 835. Wells, J. A. *Biochemistry* **1990**, *29*, 8511).

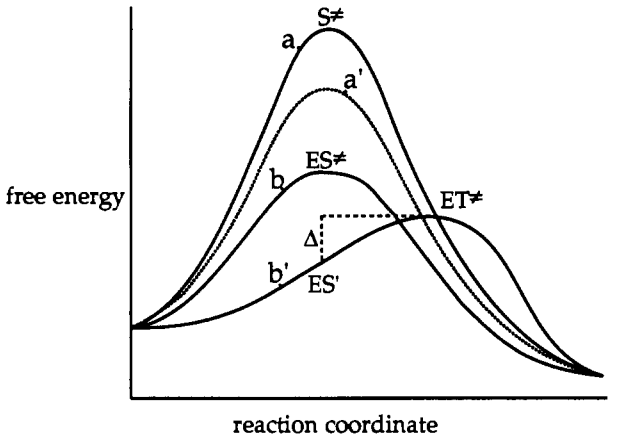

Figure 2. Progress curves, with ground-state levels normalized arbitrarily, for a nonenzymatic reaction (a) and an enzymatic reaction (b) proceeding through a transition state of similar structure. Progress curves are also shown for a nonenzymatic reaction (a′) proceeding by a different mechanism, which is faster than the reaction proceeding by mechanism a, and an enzymatic reaction (b′) proceeding by a mechanism resembling that of a and b, except that its transition state has shifted to the right. Chemical intermediate ES′ is then more stable than the shifted transition state for this reaction, by the amount Δ.

droxyl groups to be determined directly rather than by inference from substrate reactivities. For example, leucine aminopeptidase is strongly inhibited by the hydrate of leucine aldehyde and by the antibiotic amastatin (XII and XIII, Figure 1). Each of these molecules is believed to resemble an adduct formed by direct water attack on the peptide bond, and in each case, removal of a single hydroxyl group reduces the equilibrium binding affinity of the inhibitor by a factor of more than 1000 (XII and XIII, Table I).

Hydrolytic deamination of adenosine, catalyzed by fungal and mammalian enzymes, is strongly inhibited by analogues of an unstable hydrate intermediate formed by 1,6-addition of substrate water approaching from the front side of the adenosine ring as viewed in Figure 3. Thus, 6-(hydroxymethyl)-1,6-dihydropurine ribonucleoside (HDHPR) and the antibiotics coformycin and 2′-deoxycoformycin are powerful competitive inhibitors. Crystallographic studies show that the critical hydroxyl group of the hydroxymethyl substituent of the active isomer of HDHPR can be superimposed on the ring hydroxyl group of the natural 8-(*R*)-OH isomer of 2′-deoxycoformycin,[9] both compounds being similar in structure to the postulated intermediate in the catalytic process. Purine ribonucleoside resembles the substrate adenosine except for replacement of the leaving NH_2 group by hydrogen and was considered until recently to be bound by adenosine deaminase as a simple competitive inhibitor with an affinity similar to the apparent affinity of the substrate. That view became untenable when ^{13}C NMR studies revealed[10]

(9) In a remarkable display of steric discrimination, adenosine deaminase binds the natural 8(*R*)-OH isomer of 2-deoxycoformycin more tightly than the synthetic 8*S* isomer by a factor of 10^7 (Schramm, V. L.; Baker, D. C. *Biochemistry* **1985**, *24*, 641). This difference in affinities might arise from strong attraction of the 8*R* isomer by the active site, from steric hindrance of binding of the 8*S* isomer, or from some combination of these effects. In the 8*S* isomer, the critical hydroxyl group projects from the back side of the ring, from which the leaving group is believed to depart during the catalytic process. The lack of specificity of this enzyme with respect to leaving groups (NH_2, CH_3NH_2, Cl, and CH_3O are similar in reactivity) suggests that the first of these explanations is likely to be correct.

(10) Kurz, L.; Frieden, C. *Biochemistry* **1987**, *26*, 8450.

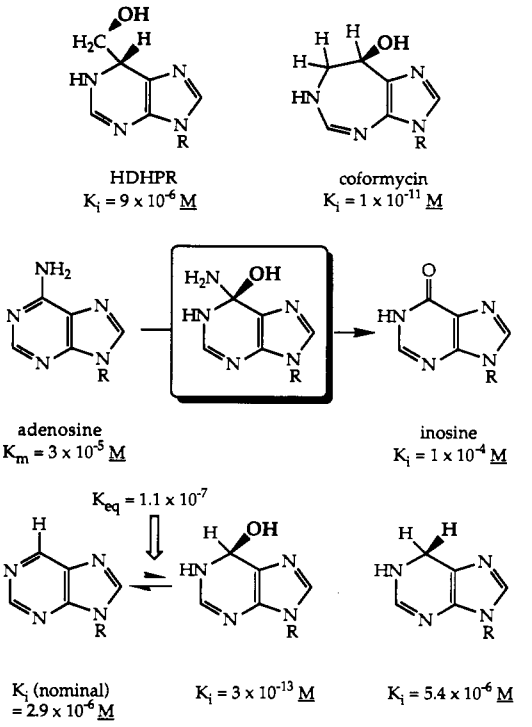

Figure 3. Binding affinities of ligands of calf intestinal adenosine deaminase.

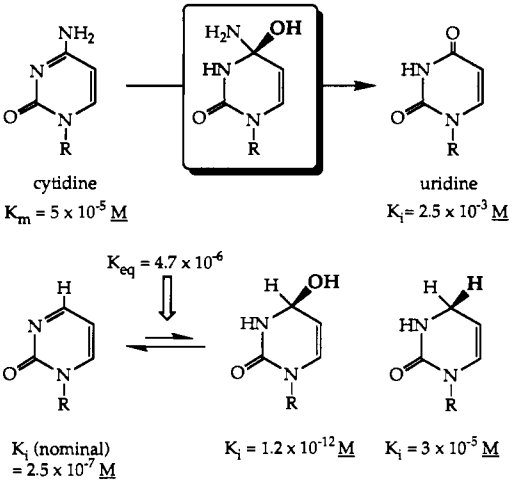

Figure 4. Binding affinities of ligands of *Escherichia coli* cytidine deaminase.

that purine ribonucleoside was bound by adenosine deaminase with a change of hybridization from sp^2 to sp^3 at C-6. NMR and UV spectra confirmed identification of enzyme-bound purine ribonucleoside as an oxygen adduct, presumably a 1,6-hydrate that is closely analogous in structure to the 1,6-hydrated intermediate in direct attack by water at the 6-position of adenosine.[11] In this structure, a hydrogen atom occupies the position presumed to be occupied by the leaving NH_2 group in the normal reaction, and because the enzyme is nonspecific with respect to the nature of this leaving group (Cl and $NHCH_3$ are similarly reactive), it is also presumably indifferent to substitution by hydrogen at this position. If the apparent K_i value of purine ribonucleoside is combined with its extremely unfavorable equilibrium constant for hydration in free solution (K_{eq}

(11) Jones, W.; Kurz, L. C.; Wolfenden, R. *Biochemistry* **1989**, *28*, 1242.

= 10^{-7}), then the true K_i value of the more inhibitory of the two diastereomers of the 1,6-hydrate is found to be in the neighborhood of 3×10^{-13} M.[12]

Cytidine deaminases from bacteria and mammals are strongly inhibited by 3,4,5,6-tetrahydrouridine, structurally analogous to a hypothetical intermediate formed by 3,4-addition of water to the alternate substrate 5,6-dihydrocytidine (Figure 4). The competitive inhibitors pyrimidin-2-one ribonucleoside (K_i(app) = 3.6×10^{-7} M) and 5-fluoropyrimidin-2-one ribonucleoside (K_i(app) = 3.5×10^{-8} M) exhibit UV absorption spectra, in their complexes with the enzyme, that are virtually identical with those of the products obtained when hydroxide ion combines with analogues quaternized at N-3.[14] These results indicate that the bound inhibitors are oxygen adducts and provide evidence in favor of binding as a covalent hydrate, not as an enzyme cysteine derivative that had been considered as an alternative possibility. The apparent K_i value of pyrimidin-2-one ribonucleoside as an inhibitor of bacterial cytidine deaminase, combined with its equilibrium constant for covalent hydration in free solution, indicates that K_i = 1.2×10^{-12} M for 3,4-dihydrouridine (the 3,4-hydrate of pyrimidin-2-one ribonucleoside).

Adenosine and cytidine deaminases are nonspecific in their action with respect to the leaving group in substrates, so that they are probably indifferent to replacement of the leaving group by hydrogen in analogues I and II and bind these transition-state analogues very tightly (Figure 1). Thus, the hydroxyl group at the sp^3-hybridized carbon atom probably offers one of the few structural features that could be used by either adenosine or cytidine deaminase to distinguish the altered substrate in the transition state for deamination, from the substrate in the ground state (Figures 3 and 4). To assess the contribution of this hydroxyl group to the binding of analogues I and II, we examined the results of its replacement of hydrogen. 1,6-Dihydropurine ribonucleoside was prepared photochemically and found to serve as a simple competitive inhibitor of adenosine deaminase, with $K_i = 5.4 \times 10^{-6}$ M. When this value was compared with the K_i value of the 1,6-hydrate of purine ribonucleoside (1.6×10^{-13} M), it became evident that the 6-hydroxyl group of the latter compound contributes −9.8 kcal/mol to the free energy of its binding by calf intestinal adenosine deaminase (Figure 3).[15,16] Similar experiments on bacterial cytidine deaminase, performed with 3,4-dihydropyrimidin-2-one ribonucleoside ($K_i = 3.0 \times 10^{-5}$ M), showed that the 4-hydroxyl group of 3,4-dihydrouridine contributes −10.1 kcal/mol to its free energy of binding (Figure 4).[14] Molecular orbital calculations suggest that the geometry and density of electrons are essentially identical at other positions in the hydrogen- and hydroxyl-substituted ligands, so that these hydroxyl group contributions to binding affinity, in the neighborhood of −10 kcal/mol, can be considered to result from simple replacement of OH by H.

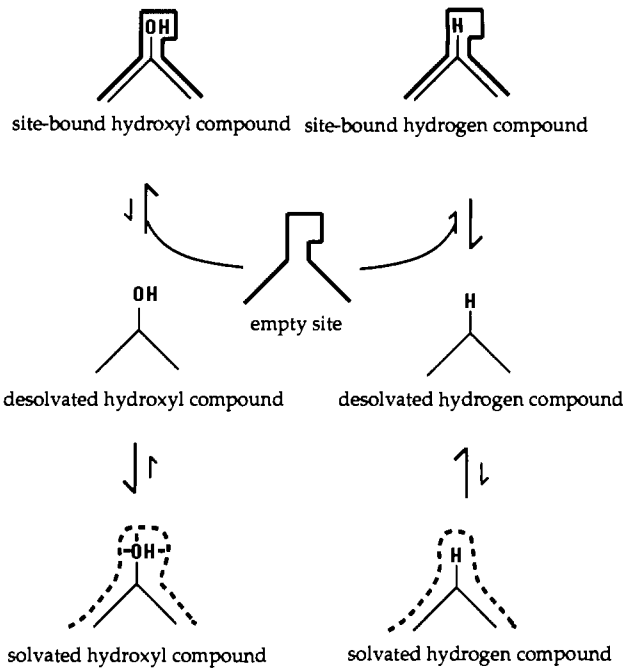

Figure 5. Analysis of equilibria of binding of hydroxyl- and hydrogen-containing ligands by a common active site, in terms of equilibria of desolvation and equilibria of binding of desolvated ligands.

Influences of Solvent Water on Observed Binding Contributions

When it enters a protein, a ligand must normally be removed, at least in part, from solvent water. To compare the inherent affinities of the desolvated ligands for the active site, it would therefore be of interest to correct for the free energies of prior removal of a hydroxyl-containing and a hydrogen-containing ligand from solvent water as shown in Figure 5. (Binding also involves removal of the active site from its previous contact with solvent water, but this is true in either case and does not contribute to the *difference* in affinities between the hydroxyl-containing and the hydrogen-containing ligands.) Free energies have now been determined for removal of many compounds of biological interest from solvent water, by measuring their water-to-vapor distribution coefficients.[17] To a fair approximation, free energies of solvation of organic compounds are found to vary as an additive function of their constituent groups, alcohols being solvated more strongly than the corresponding alkanes by a factor of roughly 10^5. If a hydroxyl-containing ligand is more readily desolvated than the corresponding hydrogen-containing ligands by roughly 7 kcal/mol in free energy, then for both adenosine and cytidine deaminases, the contribution of a desolvated hydroxyl group to the binding of a transition-state-analogue inhibitor appears to be in the neighborhood of −17 kcal/mol.

In arriving at this conclusion, we have assumed that solvent water has been stripped completely from ligands at their critical points of contact with the enzyme. That assumption, although it seems reasonable for the hy-

(12) Jones, W.; Wolfenden, R. *J. Am. Chem. Soc.* **1986**, *108*, 7444.

(13) From the rate of onset of inhibition and the rarity of the hydrate in free solution, it is clear that inhibition normally occurs as a result of purine ribonucleoside binding, followed by hydration at the active site in a mockery of the normal catalytic process.[10,12] The equilibrium of hydration appears to be greatly enhanced at the enzyme's active site, where the effective concentration of substrate water is in the neighborhood of 10^{10} M.[11]

(14) Frick, L.; Yang, C.; Marquez, V. E.; Wolfenden, R. *Biochemistry* **1989**, *28*, 9423. Similar changes in the UV spectrum have also been reported for cytosine deaminase from yeast (Kornblatt, J.; Tee, O. S. *Eur. J. Biochem.* **1986**, *156*, 297).

(15) Kati, W. M.; Wolfenden, R. *Science* **1989**, *243*, 1591–1593.

(16) Kati, W. M.; Wolfenden, R. *Biochemistry* **1989**, *28*, 7919.

(17) Wolfenden, R. *Science* **1983**, *222*, 1087.

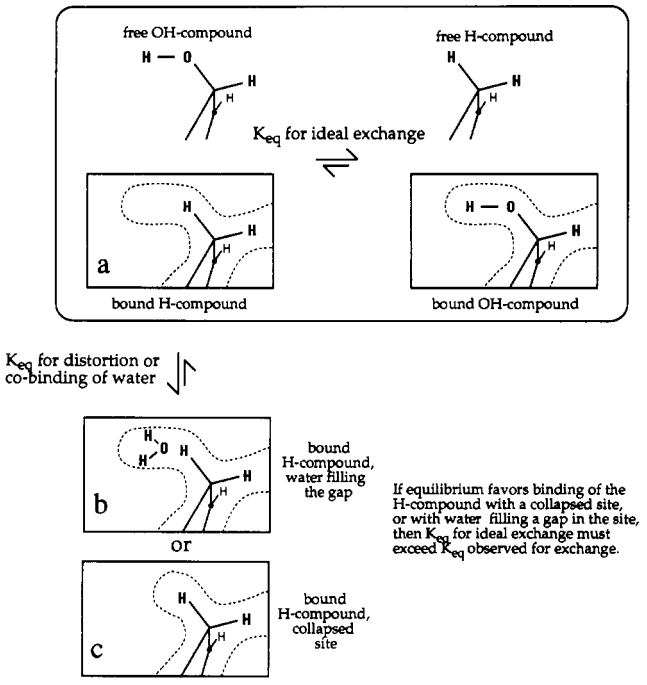

Figure 6. (a) Equilibrium constant for ideal exchange of a hydroxyl- for a hydrogen-containing ligand, with potential complications arising from (b) water trapping or (c) distortion of the active site.

droxylated ligand whose high affinity implies a close fit to the active site, may not be appropriate in the case of the hydrogen-containing ligand. In the latter case a molecule of water may take the place of the missing hydroxyl group as shown in Figure 6. This "trapping" of water would invalidate simple comparison of observed binding affinities as a measure of the contribution of the hydroxyl group to binding affinity. However, if water is in fact trapped in this way, then the stability of the resulting "wet" complex of the hydrogen-containing ligand (c, Figure 6) must presumably be greater than that of any hypothetical "dry" complex of the hydrogen-containing ligand (a, Figure 6), from which trapped water was absent. Otherwise, a "dry" complex, of the kind needed for direct comparison of binding affinities, would have been formed by the hydrogen-containing ligand. Under these circumstances, the observed difference in binding affinities would be less than the difference in "dry" binding affinities that is needed, in order to determine the contribution of the hydroxyl group to ligand binding.

The meaning of our estimate of the contribution of the critical hydroxyl group to binding, based on the difference in binding affinity between the two ligands, would also be clouded if the enzyme's conformation were to change and, to a different extent, upon binding of the different ligands.[18] The high affinity observed for the hydroxylated ligand suggests that the enzyme's native conformation is well-adapted to tight binding of the hydroxyl-containing ligand. The hydrogen-containing ligand, being smaller, should be able to fit into any "native" structure that can accommodate the hydroxylated ligand. It would hardly be surprising, however, if the enzyme's active site were to show some tendency to collapse around the hydrogen-containing ligand, forming a more compact structure than does the complex of the hydroxyl-containing ligand. Such a change in structure would invalidate simple comparison of binding affinities as a measure of hydroxyl group contribution to binding. If, however, the hydrogen-containing ligand *were* bound with such a change in conformation, then the stability of the resulting "collapsed" complex (b, Figure 6) would necessarily be greater than that of any complex with the active site in its "native" configuration (a, Figure 6). Otherwise the natively configured complex, being more stable, would have been the species actually observed at equilibrium. The hydroxyl group's contribution to the stability of the complex of the hydroxyl-containing ligand in the native structure would again have been underestimated.

These considerations suggest that if "water trapping" or enzyme distortion accompanies formation of the enzyme's complex with the hydrogen-containing ligand, then either of these effects might be expected to exert a "leveling" influence on the relative affinities observed for the hydroxyl- and hydrogen-containing ligands, leading to underestimation of the contribution of the critical hydroxyl group to binding affinity. Accordingly, the contributions of these hydroxyl groups to binding affinities of the desolvated ligands are probably at least as large as, and could be larger than, values in the neighborhood of −17 kcal/mol, suggested by the observed differences in K_i values.

Origins of Hydroxyl Group Binding Discrimination

Wilson et al. have determined the structure of the complex formed between adenosine deaminase and purine ribonucleoside at a resolution of 2.4 Å.[18] The results confirm that this inhibitor is bound as the covalent hydrate with C-6 in the absolute configuration in Figure 3. In addition, the bound inhibitor is almost completely removed from contact with solvent water, as in the complex that is formed between another transition-state-analogue inhibitor, 2-phosphoglycolate, and triosephosphate isomerase.[19] The almost complete enclosure of these analogues by the active site implies the existence of a conformation change following substrate binding. Such a conformation change may tend to maximize the possibility of attractive interactions between the enzyme and the substrate in the transition state, helping to answer the conflicting requirements of transition-state stabilization and rapid access of substrates and egress of products.[6]

Several features of the new crystal structure that are important for the present discussion are shown schematically in Figure 7. The critical 6-hydroxyl group of the inhibitor, on which so much of the catalytic binding enhancement appears to depend, interacts with a zinc atom, with a protonated histidyl residue, and with an aspartic acid residue at the enzyme's active site.

It is of interest to consider the potential magnitudes of the contributions made by these interactions to the overall binding affinity of the inhibitor. Because of the polarity of the medium, individual electrostatic or H-bonding interactions are too weak to be observed in solvent water.[20] In the absence of water, however,

(18) Wilson, D. K.; Rudolph, F. B.; Quiocho, F. A. *Science* **1991**, *252*, 1278.

(19) Lolis, E.; Petsko, G. A. *Biochemistry* **1990**, *29*, 6619.
(20) Stahl, N.; Jencks, W. P. *J. Am. Chem. Soc.* **1986**, *108*, 572.

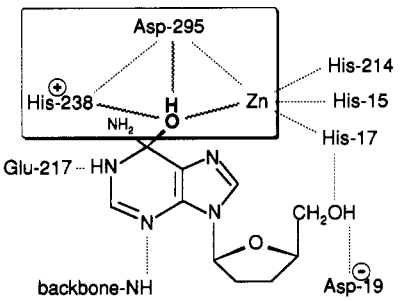

Figure 7. Some enzyme interactions with a tetrahedral intermediate in deamination of adenosine, inferred from the crystal structure of the inhibitory complex formed between adenosine deaminase and 6-hydroxy-1,6-dihydropurine ribonucleoside.[18] In ref. 18, Asp-295 is depicted as uncharged, having abstracted a proton from the attacking water molecule. At pH 4.2, where crystals were grown, adenosine deaminase is largely inactive and analogue II is relatively weakly bound. At neutral pH, where the enzyme is most active, the complex presumably contains one less proton. It may therefore be appropriate to consider the possibility that in the activated complex, Asp-295 bears a negative charge, His-238 (rather than Asp-295) having served as the general base that abstracted a proton from the attacking water molecule.

formation of a single bond between uncharged partners is accompanied by enthalpy changes of approximately −7 kcal/mol for —OH···O=C< and −5.7 kcal/mol for —OH···O<. These values, estimated from second virial coefficients observed for acetic acid[21] and water[22] vapors over water, are roughly matched by values that have been estimated by using molecular mechanics simulations.[23] Very much stronger bonds are formed when one of the partners bears an electrostatic charge. By measuring equilibria of cluster ion formation in the vapor phase,[24,25] Moet-ner and his associates have been able to demonstrate that enthalpies of formation of single hydrogen bonds between water and substituted ammonium or carboxylate ions range from −14.5 to −19.6 kcal/mol. It seems reasonable to suppose that negative enthalpies at least this large, and perhaps larger, might be observed for the formation of complexes between zinc and uncharged oxygen atoms.

To compare these values with the enthalpies of binding of hydroxyl- and hydrogen-containing ligands by adenosine deaminase, we measured the influence of changing temperature on equilibria of binding and covalent hydration of purine ribonucleoside, and on the equilibrium of binding of 1,6-dihydropurine ribonucleoside. Entropies of binding of these two ligands were found to be closely similar, but the enthalpy of binding of 6-hydroxy-1,6-dihydropurine ribonucleoside was more favorable than that of 1,6-dihydropurine ribonucleoside by 9.8 kcal/mol.[16] Earlier work had shown that alcohols are solvated by water with enthalpies that are approximately 8.2 kcal/mol more negative than those of the corresponding alkanes.[26] Correcting for these differences in enthalpy of solvation, the apparent contribution of the critical hydroxyl group to the enthalpy of binding of the desolvated covalent hydrate of

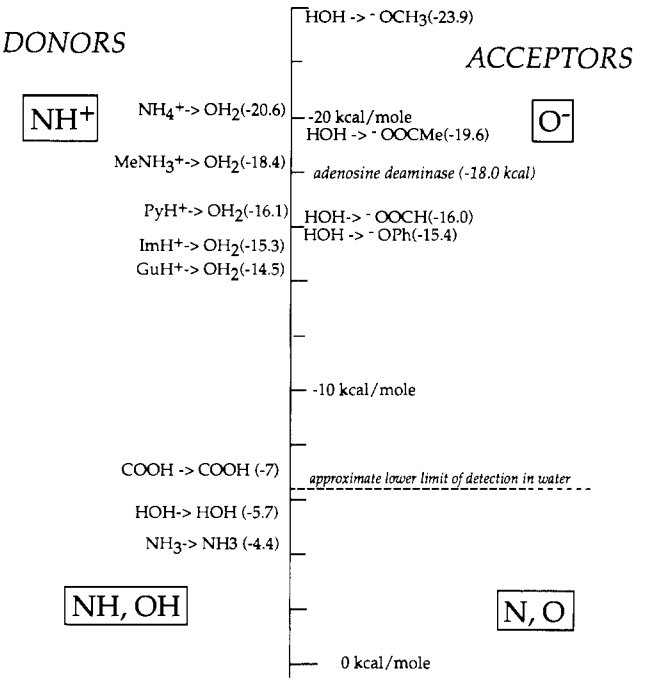

Figure 8. Enthalpies of H-bond formation in the vapor phase determined from cluster ion experiments (Meot-Ner[24] and Meot-Ner and Sieck[25]) and second virial coefficients (Lambert, J. D. *Discuss. Faraday Soc.* **1953**, *15*, 226).

purine ribonucleoside was found to be approximately −18 kcal/mol.

These comparisons, shown in Figure 8, suggest that it should not be difficult to achieve the high levels of binding discrimination that are observed in deaminases by the presence of two electrostatic interactions between the ligand's critical hydroxyl group and charged residues at the enzyme's active site. In a vacuum, even one such interaction might have been sufficient. However, in a real active site, accessory polar residues may tend to interfere with the full expression of individual bond strengths, by competing interactions with the binding partners. It is also important to recall that the contribution of the critical hydroxyl group to the enthalpy of binding was estimated only as a lower limit, as discussed in the preceding section.

Our discussion has been confined to ligand hydroxyl groups, but there is no reason why other substituents, charged or uncharged, should not be found to make contributions to binding affinity that are at least as large as those recorded in Table I, under favorable conditions.[27] These conditions seem likely to include the presence of the critical substituent in a relatively rigid ring system, so that its contribution to the overall binding affinity of the molecule involves no major loss of rotational entropy. Most of the hydroxyl groups in Figure 1 form part of such a system.

Evolutionary Aspects of Binding Discrimination: Are Enzymes Unique?

In a typical enzyme reaction, many of a substrate's structural features remain unchanged as it passes from

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(22) Christian, S. D. *J. Phys. Chem.* **1957**, *61*, 1441.
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(24) Meot-Ner, M. *J. Am. Chem. Soc.* **1984**, *106*, 1257.
(25) Meot-Ner, M.; Sieck, L. W. *J. Am. Chem. Soc.* **1986**, *108*, 7525.
(26) Butler, J. A. V. *Trans. Faraday Soc.* **1937**, *33*, 229.

(27) For example, a vanadium-containing substituent at the 6-position of glucose 1-phosphate confers extraordinary stability on its inhibitory complexes with phosphoglucomutase (Percival, M. D.; Doherty, K.; Gresser, M. J. *Biochemistry* **1990**, *29*, 2764. Ray, W. J., Jr.; Puvathingal, J. M. *Biochemistry* **1990**, *29*, 2790); and a phosphoramide group greatly strengthens the interaction of inhibitors with thermolysin (Bartlett, P. A.; Marlowe, C. K. *Science* **1987**, *235*, 569).

the ground state to the transition state. Enzymes must therefore single out for chemical recognition those few features of a substrate that do change. We have considered the generation of hydrates I and II at the active sites of deaminases as analogues of the process by which the enzyme generates intermediates in substrate hydrolysis. In these compounds, a tetrahedrally oriented hydroxyl group is an obvious feature that distinguishes these compounds from the aromatic starting materials. Evidently one or a few polar interactions involving this group, arising fleetingly in the transition state, are capable of generating a large part of the added binding affinity that is needed to explain the rate enhancement (ca. 10^{12}-fold)[28] that an enzyme of this kind produces.

Extreme levels of binding discrimination should be feasible for proteins other than enzymes, and it is of interest to consider whether there is likely to have been selective pressure for their emergence in nonenzymatic processes. For example, it should be physically possible for antibodies to develop very high affinities for antigens; indeed, prospects are encouraging that this can be accomplished by chemical or genetically induced modification of monoclonal antibodies. In experimental animals, however, few antibodies have been reported with affinities corresponding to dissociation constants of less than 10^{-10} M. This appears natural if one considers that, in an immunized individual, concentrations of circulating antibodies are typically 10^{-8} M or higher, and that these antibodies should be sufficient to "titrate" any ligand with a dissociation constant much lower than 10^{-8} M. Because removal of the antigen is already so efficient, a complex with a dissociation constant of 10^{-11} M probably offers little selective advantage over a complex with a dissociation constant of 10^{-10} M.

It is also of interest to consider the range of binding affinities that is likely to be useful in proteins that serve a regulatory function. In controlling the activity of an allosteric enzyme, for example, it is presumably necessary that ligand binding take place reversibly on a biological time scale, allowing the ligand to be bound and released at a sufficient rate to respond to changing conditions. A regulatory complex with a dissociation constant of 10^{-13} M, because of its slow rate of ligand release, would require hours to arrive at binding equilibrium and would therefore appear unsuitable for regulation over short periods of time.[29] Enzyme–substrate complexes escape this difficulty, because the binding forces that fleetingly stabilize the transition state are not yet present in the enzyme–substrate complex and are no longer present in the enzyme–product complex. With the exergonic, monomolecular collapse of $ES^{\ddagger}$ to EP, bonds that were critical for transition-state stabilization vanish, removing what would otherwise be formidable kinetic barriers to the entry of substrates and the egress of products.[30]

We are grateful to Walda Jones Powell, Lloyd Frick, and Charles Yang for their experimental and theoretical contributions to this work. Work in this laboratory was supported by NIH Grant No. GM-18325.

(28) Frick, L.; Mac Neela, J. P.; Wolfenden, R. *Bioorg. Chem.* 1987, *15*, 100.

(29) To respond to ligand concentrations changing in this range, such a "receptor" protein would itself presumably need to be present at extremely low concentrations in order to avoid removing virtually all the regulating ligand from solution.

(30) It is sometimes suggested that an enzyme could act by combining with an activated form of the substrate, which might approach the transition state in structure, rather than with the substrate in the ground state. However, any enzyme can be considered to approach the point of greatest usefulness if, among other characteristics, its second-order rate constant for product formation, k_{cat}/K_m, approaches the limit imposed by the rate at which the most abundant of the enzyme and the substrate encounter each other in solution. That criterion cannot be met by reactions between species that are not fairly populous, simply because encounter is too infrequent. From the large second-order rate constants (k_{cat}/K_m) that have been recorded for many enzyme reactions, it seems clear that mass transfer tends to occur as a result of productive combination of an enzyme with its substrate in forms that are not chemically activated to any great extent. Evidently activation must occur in synchrony with the development of strong binding forces, which relax later as products are formed and released.[6]

The Overlap Component of the Stereoelectronic Factor. Remote Control of Stereogenicity Transfer through the Anisotropic Influence of a Ring

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Received May 9, 1991 (Revised Manuscript Received June 20, 1991)

In a more innocent time, before the theory of orbital symmetry conservation, organic chemists frequently invoked the "stereoelectronic factor" in explicating and predicting reactions.[1a-c] By definition,[1b] the stereoelectronic factor causes reactions to "proceed best when certain spatial relationships pertain between electrons involved in the bonds formed or broken". These

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(1) Several good examples are described by Eliel: Eliel, E. L. *Stereochemistry of Carbon Compounds*; McGraw-Hill: New York, 1962; (a) p 139; (b) p 227; (c) pp 241–243. (d) In fact, the two ideas sometimes are not readily separable. For example, the preference for a linear rather than an angular S_N2 transition state can be explained as an orbital symmetry effect.[1e] (e) Salem, L. *Electrons in Chemical Reactions*; Wiley-Interscience: New York, 1982; p 164 and references cited therein.

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