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23. Molecular orbital studies that used energy component analyses (*17*) and added the effect of the environment would lead to a similar result.
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26. The enzyme will try to reorient its dipoles, and this reorientation is captured accurately in our free energy calculations, but the final result will involve an uphill process, as an effective enzyme is preorganized to stabilize the ionic state.
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Response: Quantum mechanics contains no forces other than coulombic, so all of molecular science in some sense "can probably be interpreted as simply electrostatic." It is not useful to do so. Most chemists prefer to reserve the term "electrostatic" for first-order coulombic interactions; that is, for those interactions all aspects of which can be understood by looking at the coulombic interaction of the groups involved, without changing the wave functions determined for them in isolation. By this criterion strong hydrogen bonds are not simply electrostatic. They give rise to characteristic changes in electronic spectrum (*1*), vibrational spectrum (*2*), and nuclear magnetic resonance spectrum (*3*). These spectroscopic changes can only be understood if the electronic and vibrational wave functions of the hydrogen bonded partners are changed by the formation of the hydrogen bond. Further, there is no generally agreed way to partition the binding energy of atoms whose van der Waals radii overlap. Thus, the statement that the interaction is electrostatic because the binding energy can be mimicked with an electrostatic calculation containing arbitrary parameters is misleading. It is based on the wave functions of the interacting system, not the isolated reactants, and on a particular partitioning scheme. It should be noted that Gilli (*4*) has concluded that as "the O · · · O distance is shortened from 2.80 to 2.40 Å, the hydrogen bond is transformed from a dissymetrical O–H · · · O electrostatic interaction to a covalent and symmetrical O · · · H · · · O bond."

Warshel *et al.* argue that hydrogen bonds in an enzyme active site will be weak because of solvation effects, and imply that this site resembles a polar solvent. It is not easy to define the strength of a hydrogen bond. In the case of a covalent bond, the diatomic interaction between the bonded atoms is so much stronger than all secondary interactions that the homolytic dissociation enthalpy provides a satisfactory measure of bond energy for most purposes. Hydrogen bonds are weaker, and their strength is sensitive to the donor-acceptor distance, as is repulsive energy. We propose the following definition for hydrogen bond strength in an enzyme: With the heavy atom geometry adjusted so that the enzyme can perform its catalytic function, the hydrogen bond energy is the increase in Gibbs free energy that would occur if the hydrogen bond were deleted.

With this definition in mind, we note that the pK_a (negative logarithm of the acidity constant) of 1,8-bis(diethylamino)-2,7-dimethoxynaphthalene is about 16.3 (*5*). That's more basic, by a factor of 10^{12}, than a simple analog with only one basic site. Converting that to free energy gives 16.5 kcal/mol, in aqueous solution. We are all agreed that the presence of water tends to attenuate hydrogen bond strength. Also, the hydrogen bond in question is between two nitrogens, which generally do not give hydrogen bonds as strong as those between oxygens. The hydrogen bonds in enzymes usually involve at least one oxygen, and they are often formed in a much less attenuating matrix than an aqueous solution. We think these observations support our statement that hydrogen bond strength in enzymes may reach 20 kcal/mol in favorable cases. We do not dispute that a good deal of this can be mimicked by an electrostatic calculation, especially if the dielectric constant and the interatomic distances are not exactly known, and can be suitably adjusted. However, the spectroscopic evidence cited above indicates that such a calculation misrepresents the physics. Further, it is generally agreed by enzymologists that an enzyme active site is not equivalent to aqueous solution, and that one important role of the conformation changes that set up catalysis is to squeeze most of the water out of the active site. Unlike a solvent, the enzyme-substrate interactions that Warshel *et al.* refer to can be evolved to stabilize a distributed charge as well as a localized charge.

Warshel *et al.* quote a recent paper [reference (*18*) in their comment] as indicating that a very short hydrogen bond in an enzyme-inhibitor complex is not a strong one. This is a misinterpretation of the data. First, the measured dissociation constant of the inhibitor was not extrapolated to a pH where the inhibitor is protonated, and this must be done to get the true dissociation constant (the inhibitor binds with its carboxyl protonated). Second, there is no way to determine the degree to which the changes from substrate to inhibitor have *decreased* affinity. If allowance were made for such effects, the low barrier hydrogen bond that appears to be present would be producing a large amount of binding energy.

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Response: Tobin, Whitt, and I did not state in our report that hydrogen bond stabilization corresponding to 30 kcal/mol is realized in catalysis by serine proteases. We raised the question of how strong the LBHB between His^{57} and Asp^{102} is, and we specifically excluded 30 kcal/mol. Further, we pointed out that the decrease in rate upon disruption of the LBHB corresponds to 10^4 to 10^5, or 5 to 7 kcal/mol in activation energy. This is an important amount of catalysis. The statement by Warshel *et al.* that all hydrogen bonding effects in condensed phases are weak electrostatic effects is not supported by experimental observations.

1) Microenvironments at enzymatic active sites.

Warshel *et al.* state that hydrogen bonds in condensed phases are strictly weakly electrostatic in nature and that there are no strong hydrogen bonds. Their computational models for enzymes assign an essentially liquid solvent state to active sites. This is an arbitrary assumption. Evidence that substrate molecules are desolvated at active sites is overwhelming. The nonliquid nature of enzyme surfaces is evidenced most simply by the presence of hundreds of fixed water molecules at the water-protein interface of any enzyme. Bulk water is generally excluded from the interiors of enzymes, where the packing densities are 0.7 to 0.8, or approximately

Response

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