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Low-Barrier Hydrogen Bonds and Low Fractionation Factor Bases in Enzymatic Reactions†

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The deuterium fractionation factor of a hydrogen in a molecule is the tendency for deuterium to enrich in this position relative to its content in water. Thus, an exchangeable hydrogen with a fractionation factor of 0.5 contains half the deuterium content of solvent water as long as only traces of deuterium are present. Carbon-bonded hydrogens that are not exchangeable except when removed during a reaction have fractionation factors ranging from 0.64 for acetylene to 1.23 for hemiacetals such as the hydrogen at C-1 of glucose (Cleland, 1980). The sulfhydryl group is the only part of a normal amino acid side chain which has a hydrogen exchangeable with the solvent with a fractionation factor less than unity (~0.5; Schowen, 1972). Carboxyl, imidazole, amino, and hydroxy groups all have fractionation factors close to unity.

Thus, when experiments have indicated that the fractionation factors of catalytic groups on enzymes were ~0.5, the supposition has been that these were sulfhydryl groups. This is probably the case with proline racemase (Rudnick & Abeles, 1975; Belasco et al., 1986) and possibly with the related hydroxyproline racemase (Finlay & Adams, 1970), but in the absence of structures from X-ray studies there is still some uncertainty.

The present author has postulated that enolase has a sulfhydryl group as the base that deprotonates 2-phosphoglycerate to give the carbanion intermediate, since the dissociation constant of 2-deuterated tartronate semialdehyde phosphate (TSP) was twice that of unlabeled TSP (Weiss et al., 1987a). This inhibitor is a pseudosubstrate, and it is deprotonated on the enzyme to an enediolate with proton transfer from C-2 to the enzymic base (Spring & Wold, 1971; Lane & Hurst, 1974). The low fractionation factor of this base decreases the equilibrium constant for deuterium transfer from TSP [where the C-2 hydrogen has a fractionation factor of 1.19 (Cleland, 1980)] so that it is half what it is with hydrogen transfer.

We also thought adenosine deaminase had a sulfhydryl group donating a proton to N-1 of adenosine during the reaction, since in D_2O the equilibrium constant for formation of the tetrahedral intermediate where hydroxide has added to C-6 of adenosine or 8-oxoadenosine is twice as high as in H_2O (Weiss et al., 1987b). This is the expected result if the group donating the proton to N-1 has a fractionation factor of ~0.5, since the value for a proton on N-1 is close to unity.

Structures are now available from X-ray crystallography for both enolase (Lebioda & Stec, 1991) and adenosine deaminase (Wilson et al., 1991), and to our embarrassment neither indicates a sulfhydryl group as the putative enzymic base. In enolase, the base appears to be a water molecule held between two glutamates, and in adenosine deaminase the base is a (presumably protonated) glutamate.

Low fractionation factors for enzyme groups involved in acid–base catalysis have also been seen for pyruvate carboxylase (Attwood et al., 1986) and biotin carboxylase (Tipton & Cleland, 1988), but X-ray structures are not yet available for these enzymes.

So why are the fractionation factors of bases on enolase and adenosine deaminase so low? We would perhaps not have been so hasty to invoke sulfhydryl groups if we had paid more attention to the work of Kreevoy (Kreevoy et al., 1977; Kreevoy & Liang, 1980) on low-barrier hydrogen bonds. To understand what these are, let us consider the structure of a hydrogen bond between two groups with equal pK values:

$$R\text{–}O\text{–}H\cdots O\text{–}R' \rightleftharpoons R\text{–}O\cdots H\text{–}O\text{–}R'$$

At an O–O distance of >2.8 Å, the potential function for this system describes a double well, with the hydrogen belonging either to R–O or to O–R′, and an appreciable barrier between the two wells (Figure 1A). The fractionation factor of such a proton is near unity (when it is attached to oxygens as shown), since this is the type of hydrogen bond that exists in water. As the O–O distance is compressed, the barrier between the two wells decreases in height, and when the distance is less than ~2.55 Å, the hydrogen can freely move between the two

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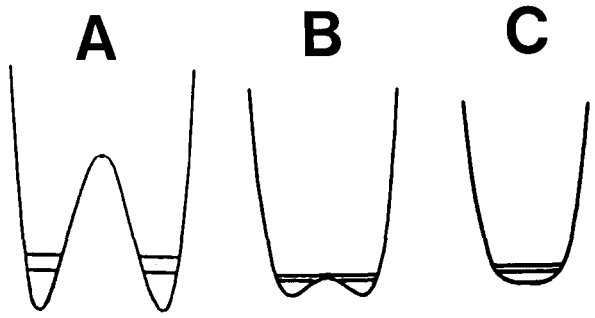


FIGURE 1: Potential functions for hydrogen bonds [after Kreevoy and Liang (1980)]. The horizontal lines are lowest energy levels for H (upper) and D. (A) Double-well hydrogen bond. (B) Low-barrier hydrogen bond. (C) Single-well hydrogen bond.

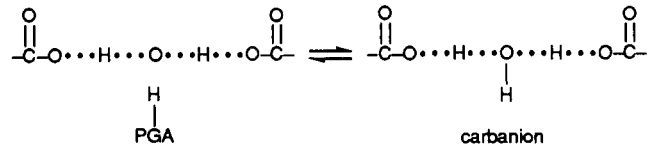
wells. Because its position now averages in the center of the O–O coordinate [as inferred experimentally by neutron diffraction (MacDonald et al., 1972; Bacon et al., 1977; Attig & Williams, 1977) and NMR studies (Altman et al., 1978)], the hydrogen is weakly bonded to either oxygen (as evidenced by very broad, low-frequency IR bands; Hadži & Bratoš, 1976), and its fractionation factor gets very low (the lowest experimental value is 0.30 for an O–O distance of ~2.45 Å). We will call this structure a low-barrier hydrogen bond (Figure 1B). As further compression of the O–O distance occurs, the center barrier disappears completely and the increasing bond order to the center hydrogen as the O–O distance shortens causes the fractionation factor to rise somewhat. We can call this a single-well hydrogen bond (Figure 1C). A well-known example of the latter is the FHF^- ion, which has a fractionation factor of 0.6 in water (Kreevoy & Liang, 1980) and an F–F distance of 2.27 Å (Ibers, 1964).

Experimental examples of low-barrier hydrogen bonds with low fractionation factors include ions in which a proton is shared between two carboxylate or two phenolate groups; the fractionation factor of the shared proton in acetonitrile as solvent can be as low as 0.30 for 3,5-dinitrobenzoate or *p*-nitrophenolate (Kreevoy & Liang, 1980). Other examples are trifluoroacetate (0.42), 3,5-dinitrophenolate (0.36), and pentachlorophenolate (0.40). A proton shared between two oxygens of dimethyl sulfoxide in that solvent has a fractionation factor of 0.36. The $H_5O_2^+$ ion present in some crystals where a proton is midway between two water molecules with an O–O spacing of 2.45 Å is another example (Bacon et al., 1977); this ion in the gas phase has a fractionation factor for the central hydrogen of 0.55 (Graul et al., 1990).

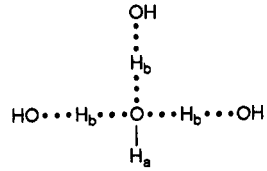
The fractionation factors of protons in symmetrical hydrogen bonds are higher in water and other protic solvents than in solvents like acetonitrile or dimethyl sulfoxide [0.84 for the proton in monoprotonated maleic acid in water, for example (Kreevoy & Liang, 1980)], since other hydrogen bonding in protic solvents presumably keeps the O–O distance from being sufficiently short to lower the central barrier very much. Thus, the fractionation factor of the central hydrogen in the $[MeO\cdots H\cdots OMe]^-$ ion in methanol is 0.74, while in 25% methanol/75% dimethyl sulfoxide it is 0.38 (Baltzer & Bergman, 1982) and in the gas phase it is 0.33 (Weil & Dixon, 1985). However, the environment in an enzyme active site is more similar to that in solvents such as acetonitrile or dimethyl sulfoxide than it is to that in aqueous solution, so whenever the p*K*'s of the partners in the hydrogen bond are similar, it is very reasonable that it would have a low central barrier.

The adenosine deaminase case is clear-cut. The X-ray structure of purine riboside bound to the enzyme shows that this pseudosubstrate has become hydrated by attack of hydroxide at C-6, and one arm of Glu-217 is 2.8 Å from N-1 (Wilson et al., 1991). Clearly, in free enzyme Glu-217 is protonated, and in the initially formed complex with adenosine the hydrogen bond between Glu-217 and N-1 of adenosine must be a low-barrier one with a low fractionation factor (~0.4). This is reasonable, since the p*K*'s of Glu-217 and N-1 of adenosine are probably very similar. Once OH adds to C-6, however, the p*K* of N-1 becomes much higher than that of Glu-217 and the proton will be firmly attached to N-1, although still hydrogen bonded to Glu-217, and have a fractionation factor near unity.

In enolase, a water molecule is seen immobilized between Glu-168 and Glu-211 in a structure that contains one Mg^{2+} ion and 2-phosphoglycerate (Lebioda & Stec, 1991). This water is in close contact (2.6 Å) with C-2 of 2-phosphoglycerate, and it appears poised to act as the general base for proton removal to give the carbanion intermediate. [Important caveats here: (1) this structure lacks the second metal ion which binds to phosphate oxygens and shares a ligand with the first metal ion (R. R. Poyner and G. H. Reed, personal communication); (2) the enzyme was crystallized at low pH, where it is inactive.] If this water is indeed the base, this part of the reaction can be diagrammed as shown:



In the left-hand structure the cluster of two glutamates and bound water has a charge of −2, while the right-hand structure has a charge of −1. If the hydrogen bonds to the glutamates are low-barrier ones, then the shared hydrogens will be very weakly bonded to the center oxygen and the fractionation factor of the hydrogen attached to the central oxygen could presumably be less than that of water because of the decreased contribution from bending vibrations. The fractionation factor of hydroxide which is thought to exist in water as a trihydrate



is 0.43 (Chiang et al., 1980), but there has been much controversy over how much of this value results from lowered fractionation factors for H_a vs the three H_b hydrogens. Gold and Grist (1972) originally assigned values of 1.2–1.5 for H_a and 0.65–0.70 for each H_b, but Kresge et al. (1987) dispute this and suggest that both H_a and H_b may have values of ~0.8. They conclude that further measurements are needed to settle the matter.

In any case, the present evidence suggests that in the nonaqueous environment of the enolase active site the fractionation factor of the hydrogen transferred from the substrate is around 0.5. Possibly, binding of the second metal ion rearranges the groups in the active site so that water is not the general base, but certainly the thiol group of cysteine is not the base, since the only cysteines in various enolases are in nonconserved positions in the sequence.

The question is, are low-barrier hydrogen bonds important for enzymic catalysis? To the extent that proton motion does not have to contribute to reaction coordinate motion, chemical steps may be accelerated. This could be the case with adenosine deaminase, where there will not be an appreciable ac-

tivation energy for the proton shift that accompanies hydroxide attack at C-6. With enolase, however, it is not clear that the low-barrier hydrogen bonds are doing anything except acting to position the bound water molecule and perhaps alter its pK. It must be remembered that low-barrier hydrogen bonds are strong hydrogen bonds, because of the short O–O distance, even though the bonding of the central hydrogen to either oxygen is weak. Another example of what are certainly low-barrier hydrogen bonds is the short glutamate to phosphonate oxygen distance of 2.2–2.5 Å seen in six separate structures of carboxypeptidase (Kim & Lipscomb, 1990, 1991) or thermolysin (Tronrud et al., 1986; Holden et al., 1987) with phosphonate inhibitors bound in bidentate fashion to active site zinc. This glutamate (270 in carboxypeptidase; 143 in thermolysin) is the general base for proton removal from water during the hydrolytic attack. Structures of aconitase with bound isocitrate or the nitro analogue of isocitrate show a 2.7-Å hydrogen bond between Fe-coordinated water and the carboxyl group of isocitrate, which becomes the aci-carboxylate during carbanion formation, and a 2.5-Å hydrogen bond in the corresponding structure with the analogue ionized to a nitronate and mimicking the intermediate carbanion structure (Lauble et al., 1992).

It is very likely that more examples of low-barrier hydrogen bonds will be found, and enzymologists and protein crystallographers should get used to thinking about them. The low fractionation factors these low-barrier hydrogen bonds display together with structural information should readily allow them to be identified. They will form, however, only when the pK values of the two groups in the hydrogen bond are similar.

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