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PAPER

Hydrogen-bond stabilization in oxyanion holes: *grand jeté* to three dimensions†

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We recently reported crystallographic evidence that the hydrogen bonds which can stabilize oxygen-centered negative charge within enzyme oxyanion holes are rarely found in the place they should be expected on the basis of the analysis of small-molecule crystal structures. We investigated this phenomenon using calculations on simplified active site models. A recent paper suggested that several aspects of the analysis required further exploration. In this paper we: (i) review the results of our crystallographic study; (ii) report molecular dynamics studies which investigate the effect of protein movement; (iii) report ONIOM calculations which trace the reaction coordinate for an oxyanion hole reaction in the presence of a complete enzyme active site. These results show that the limitations of gas phase calculations on simplified models do not invalidate our comparison of competing active site geometries. These new results reaffirm the conclusion that oxyanion holes are not usually stabilized by planar arrangements of H-bonds, and that this sub-optimal transition state stabilization leads to better overall catalysis.

Introduction

The precise details of the mechanisms of enzyme reactions are an area of continuous research and debate. A selection of recent contributions to the area[1–15] is a starting point for tracing developments in this field. We have recently published[16] a study on the preferred structures of oxyanion holes. There are many enzymes in which these motifs stabilize the developing negative charge on a reacting carbonyl oxygen by the formation of two or three hydrogen bonds.[17–26] Our study was based on the analysis of many crystal structures of enzymes containing this moiety and demonstrated that the preferred orientation is not the expected one (Fig. 1). Subsequently, Warshel *et al.*[27] analyzed our work and suggested "*the conclusions are unjustified*". In this paper we report more advanced computational studies of the phenomenon and clarify some of the statements that could lead to misinterpretation.

In our original paper, we analyzed the structure of more than three hundred crystal structures of enzymes containing oxyanion holes. These structures were gathered from the list of enzyme crystal structures contained in the Protein Data Bank[28–30] at the time of the study, using an automatic procedure based on the presence of two H-bond donors nearby a carbonyl group and on

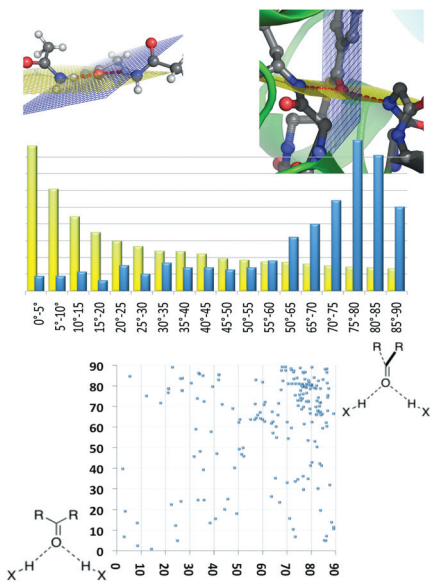

Fig. 1 Above: distribution of H-bond dihedral angles in molecules in the CSD (yellow) and in enzymes (blue); below: scatter plot of the dihedral angles in enzymes. The R–C═O···HX dihedral angle prefers values around 90° rather than 0°.

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the type of reaction catalyzed (according to their EC codes). The details of this procedure can be found in the original paper.[16]

With these oxyanion hole structures in hand, we analyzed various parameters that characterize their geometries, hoping to gain insights into the design of organocatalysts containing oxyanion hole mimics. The most striking result was the distribution of the dihedral angle between the H-bond donors and the carbonyl oxygen. This distribution, reproduced in Fig. 1 (blue bars), shows a clear preference for dihedral angles near 90°, in contrast to the corresponding pattern (yellow bars) from data in the Cambridge Structure Database,[31,32] which was obtained analyzing the crystal structures of hydrogen-bonded carbonyl compounds, which show a preference of 0° for the same dihedral angle,[33–35] and tetrahedral oxyanions, which show no clear preference.[36]

The traditional way of drawing the interaction between hydrogen bond donors and a carbonyl is planar and reminiscent of Leonardo da Vinci's Vetruvian Man (Fig. 2, left). A flat structure is easy to draw and this may be why it is so widely used as a representation of a carbonyl activated towards nucleophilic attack. However, this simple planar representation is not an accurate representation of the great majority of oxyanion holes in the PDB. A better representation of the preferred structure is closer to a *grand jeté* with arms in second position (Fig. 2, right; in ballet, a *grand jeté* is a long horizontal jump starting from one leg and landing on the other; the dancer usually extends his arms in a perpendicular position to his legs). This intrinsically 3D arrangement better reflects the true preferences of enzyme active sites.

The analysis of the crystal structure data leaves no room for doubt about the preferred arrangement of hydrogen bonding in oxyanion holes. The result is surprising because it does not appear to give maximum transition state (TS) stabilization. The preferred orientation of hydrogen bonds with respect to the sp^2 oxygen of carbonyls is well known from both crystal structure data and small molecules:[33–35,37] a flat orientation resembling the Vitruvian Man. An sp^3 oxygen has no such preferred direction.[36] The transition state between a sp^2 oxygen and the sp^3 oxygen of the anion should have some sp^2 character and so some directionality should be expected for maximum stabilization of the transition state. The PDB crystallographic data show that enzymes do not prefer the geometry that gives this expected stabilization. This observation has implications both for understanding enzyme mechanisms and for designing organocatalysts.

A recent paper[27] has questioned our analysis of these observations. The oxyanion hole analysis is based on the exploration of the crystal structures of enzymes, so the dihedral angles of the H-bonds could change during relaxation of these structures or as a consequence of the flexibility of the enzyme as the reaction proceeds. Our calculations on simplified models made use of a

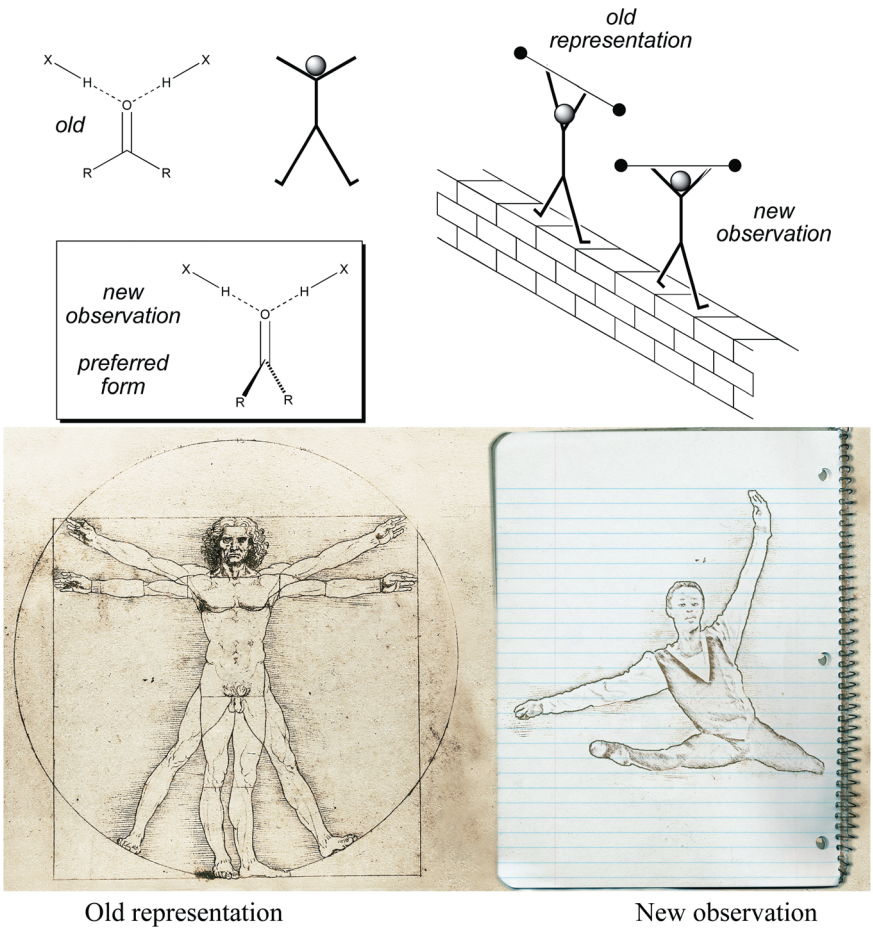

Fig. 2 Left: Vitruvian Man – the flat representation of oxyanion holes (R–C═O···HX ≈ 0°); right: *grand jeté* – a more realistic representation based on our new observations (R–C═O···HX ≈ 90°). (Pictures: left: Leonardo da Vinci, *via* Wikipedia; right: Wikimedia Commons, author: Fanny Schertzer – adapted.)

number of constraints that may have pushed the calculations towards specific conclusions. We used gas phase calculations which do not evaluate the absolute reduction of the energy barrier by the enzyme because the preorganization energy is ignored. In this paper, we investigate these issues using molecular dynamics and ONIOM calculations. We also explain how gas phase calculations can be used to rank competing enzyme reaction pathways.

Results and discussion

Does the flexibility of the protein explain the anomalous dihedral angle distribution?

The crystal structures of proteins usually have a rather low resolution (RMSD around 1.0 Å in best cases). We based our conclusions on data from nearly 200 structures and so the random errors arising from the low resolution in each individual structure should be substantially reduced. Protein crystal structures do not usually localize hydrogen atoms. The hydrogen atom positions used in our analysis were determined from the attached heteroatom. In most cases these were nitrogen atoms of amides from the protein backbone, and so the hydrogen position can be determined with considerable confidence. However, in our original study we did not consider the flexibility of the proteins nor the possibility of protein relaxing from the crystal-structure conformation. This was a shortcoming: changes in the enzyme structure during the reaction are not only possible[26] but in some cases necessary.[38] The changes in the geometry of the enzyme will contribute to the reorganization energy in the enzyme. Might the dihedral angles move through 90° to the expected position as the enzyme preorganizes toward the transition state?

Although protein structures exhibit some flexibility that is not present in the crystal structure, most of the oxyanion hole H-bond donors are part of the protein backbone and so are unlikely to be free to make the large reorganization required to nullify our conclusions from the analysis of crystal structures. This large reorganization is much more likely if the H-bond donors are not on the backbone. With this in mind, we chose to study the flexibility of subtilisin, a non-specific serine protease. One of its H-bond donors is the amide NH_2 group of an asparagine residue (Fig. 3), so reorganization and relaxation is likely to be much easier in subtilisin than in most oxyanion holes. If protein flexibility can explain the anomalous H-bonding arrangement we have observed in crystal structures, subtilisin should be one of the enzymes that shows this effect.

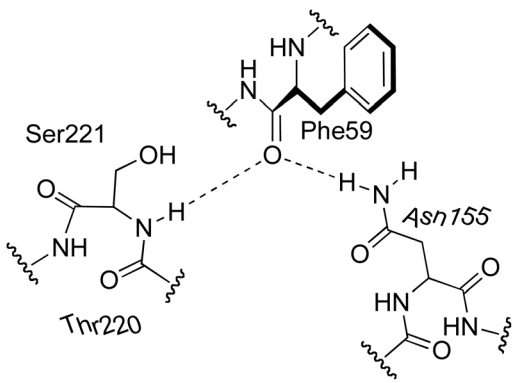

Fig. 3 Structure of the active site of subtilisin bonded to M59F mutant inhibitor. The key dihedral angles (N–C═O···HN) are between the amide of Phe59 and the N–H of Ser221 and of Asn155.

Therefore, we ran MD simulations on this enzyme to test, first, if the X-ray structure is relaxed, and second, if the geometry of the oxyanion hole can change sufficiently during the reaction to move the hydrogen bonds through 90°.

The simulations were run on the subtilisin BPN′ structure bound to chymotrypsin inhibitor 2 (PDB code: 1tmg[39] (note that this is the M59F mutant of chymotrypsin inhibitor 2 (CI2); neither methionine nor phenylalanine can form side-chain hydrogen bonds with the substrate; this mutation is likely to further decrease substrate specificity) using GROMACS 4.0[40] with the OPLSAA force field[41] in a box of water molecules at 298 K (see details in the ESI†). This combination has offered reliable results in previous studies of the flexibility of peptides.[42] After 10 ns of unconstrained simulation the dihedral angles of both H-bonds did not change to any great extent (Fig. 4(a), first 10 ns).

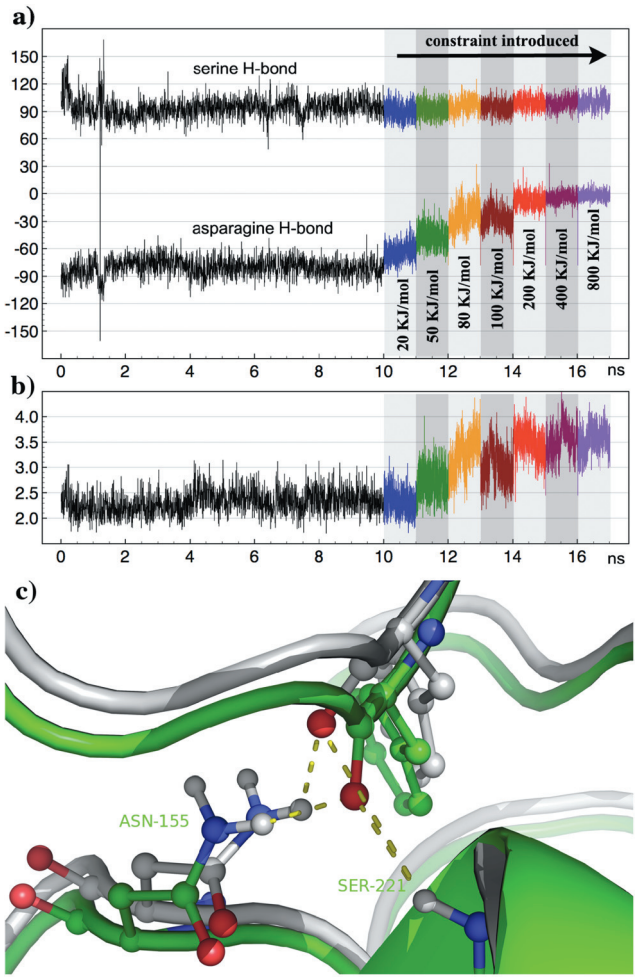

Fig. 4 a) Plot of the dihedral angle for ASN-155 H-bond and SER-221 H-bond in an unconstrained MD simulation for subtilisin and after including a 20, 50, 80, 100, 200, 400 and 800 kJ mol^{-1} rad^{-1} constraint for both H-bonds. b) Plot of the SER-221 H-bond distance during the simulation and after the addition of the constraint. c) Overlay of the oxyanion hole average structure in subtilisin during the simulation without constraint (green) and with 800 kJ mol^{-1} rad^{-1} constraint (gray) for the H-bonds.

Therefore, relaxation of the crystal structure does not greatly affect the H-bond dihedral angles even though the molecular dynamics has modified the enzyme geometry from the crystal structure. Note that in Fig. 1 we normalized the dihedral angle between 0 and 90°, whereas in Fig. 4 negative angles or angles greater than 90° appear; so both −80° or 100° in Fig. 4 were treated as 80° in Fig. 1.

Next, in order to study if an induced fit could affect these angles, harmonic constraints were introduced to push the H-bond donors towards the expected dihedral angle of 0°. If the enzyme structure changes during the reaction, some degree of flexibility would be expected, so the inclusion of the constraint should affect the dihedral angle values. We observed that the dihedral angle of the H-bond with the asparagine amide changes, but reducing it to 0° requires a 200 kJ mol^{-1} rad^{-1} constraint. In the case of the serine H-bond (using a backbone NH as donor), even after the use of a completely unrealistic 800 kJ mol^{-1} rad^{-1} constraint the angle barely changed. The constraint also affects the distance between the serine N and the carbonyl oxygen increasing it from 2.2 to 3.8 Å (Fig. 4(b)). The constraint is able to change the asparagine H-bond dihedral angle but only after breaking the serine NH H-bond. This is shown in Fig. 4(c), where the average structures without the constraint and with the 800 kJ mol^{-1} rad^{-1} constraints are overlaid. It is possible that if the asparagine H-bond donor was not in such a flexible part of the protein (as is the case of backbone NHs) both H-bonds would have been broken by the constraints.

This study of a particularly flexible enzyme demonstrates that the active site cannot be distorted to the expected 0° even when constraints of unrealistic magnitude are induced, and the H-bonds break altogether rather than twist around. We conclude, therefore, that protein flexibility does not alter the qualitative conclusions of our crystallographic study.

Insights from constrained calculations

In order to find a possible explanation for the surprising arrangement of hydrogen bonds, we ran calculations on simplified models of a serine protease in which we could study the effect of the dihedral angle of the H-bond donors in catalysis. Our calculations are similar to theozyme calculation,[43–45] in which catalytic residues in the enzyme active center are modeled as the corresponding functional groups, but instead of searching for maximum transition state stabilization, like theozyme calculations, we analyzed how changes in the geometry (*i.e.* H-bond dihedral angle) affect the catalysis. These calculations are designed to compare similar reaction pathways and not to provide estimates of the solvent reorganization energies that will be similar in all cases. (The details of these theozyme calculations are given in our previous article.[16])

The lowest energy transition state showed H-bonds with 0° dihedral angle which does not correspond to the most common dihedral angle found in the enzymes. Rather than fit the experimental data, the calculation seems to reinforce the classical point of view that enzyme catalytic activity arises from maximizing stabilization of the transition state, first suggested by Pauling[46] and widely accepted.[4,7,9,10,15,47,48] In oxyanion holes, however, the most common H-bond dihedral angle is not the one

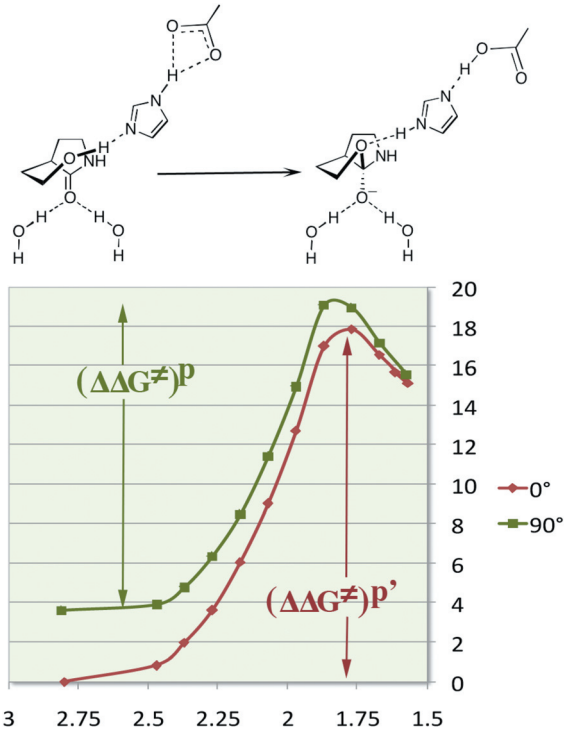

Fig. 5 Reaction profiles for the theozyme studied in our previous work forcing the H-bond dihedral angles to 90° and to 0°.

that is most effective in stabilizing the transition state, because the most stable geometry for the reactant complex has the same 0° dihedral angle, and the interaction with the reactant is more sensitive to this angle than the interactions with the TS. As a result there is an overall reduction of the barrier height for 90° H-bond dihedral angles (see Fig. 5). Topological analysis of the Laplacian of the electronic density[34] also shows that H-bonds are stronger for dihedral angles of 0°.[16]

Unlike the enzyme structure, in which catalytic groups (main chain NH hydrogen bond donors) are held in fairly fixed positions defined by the enzyme tertiary structure, the calculations of simplified models allow the initial structure to evolve and collapse during the geometry optimization leading to unrealistic active site structures. To prevent this, we included in our calculations a set of geometrical constraints, based on the parameters that we found for oxyanion hole geometries. The inclusion of these constraints leads to a more realistic situation, since it reproduces the relatively rigid geometry of the enzyme active site. However, it is also possible that the constraints have perturbed the results of the calculations, so that our explanation for the distribution of the H-bond dihedral angles arises from the constraints rather than from the underlying reactivity.[27]

In order to investigate this possibility, we have repeated the studies of the effect of the H-bond dihedral angle on a different enzyme: 4-chlorobenzoyl-CoA dehalogenase (PDB ID: 1nzy[49]). This enzyme catalyzes the hydrolytic dehalogenation of 4-chlorobenzoyl-CoA to 4-hydroxybenzoyl-CoA. The first step of the catalytic cycle consists of the attack of the carboxylic acid of the Asp145 residue to the C(4) of the substrate. The developing negative charge in the benzoyl oxygen is stabilized by the oxyanion hole formed by Gly114 and Phe64; the distance

between the H-bond donors in the oxyanion hole and the Asp145 carboxylate is long enough to prevent the interaction between the groups modeling these atoms in the theozyme, reducing the need to use constraints. We simplified the 4-chlorobenzoyl-CoA substrate with *S*-methyl 4-chlorobenzothioate and the two H-bond donors with two water molecules, which have similar H-bond donor ability to NH groups from the protein backbone[50] but are smaller and so less likely to collide with other groups in the theozyme. In order to avoid the use of arbitrary constraints, which might lead to erroneous results, we decided to keep the amino acids in the enzyme active site fixed to the Cartesian coordinates of the atom bonded to the protein backbone (Fig. 6). Because the protein backbone has a limited mobility, this was reasonable. Trp84 was not included since it could have interfered with one of the water molecules used to model the H-bond donors. For the same reason, only the phenyl ring of Phe64 was considered. The distance between the two water molecules was fixed to 4.5 Å, which corresponds to the distance between the H-bond donors in the PDB 1nzy structure, and the position of the sulfur atom in the substrate was also fixed (since otherwise the substrate left the active site during some of the calculations). We think that this theozyme not only contains a minimal number of constraints, but also that all of the constraints have reasonable molecular interpretations.

The resulting geometry was modeled using ONIOM method,[51–53] in which H-bond donors, substrate and Asp145 residue were treated with the B3LYP/6-31G**[54–56] level of theory and the remaining atoms with the AMBER force field.[57] Calculations were performed using the Gaussian03 program.[58] Embedded charges were used when calculating the wave function by the DFT method. Based on previous QM/MM simulations performed by Gao *et al.*,[59,60] the geometry of the reactant state was calculated fixing the distance between the Asp145 carboxylic oxygen atom and C(4) atom in the substrate to 3.4 Å.[60] Since it has not been possible to locate the transition state structures using conventional optimization algorithms, we performed a set of geometry optimizations in which either the C(4)–O(Asp145) distance or the C(4)–Cl distance was fixed to different values, and approximated the transition state geometry as the maximum value of the energy in the reaction coordinate path defined as the difference between the C(4)–O(Asp145) and C(4)–Cl distances. This simplified model misses some of the catalytic resources of the natural enzyme (including interactions of the molecular dipole with the Gly114–Ala 121 α-helix[59–62] and

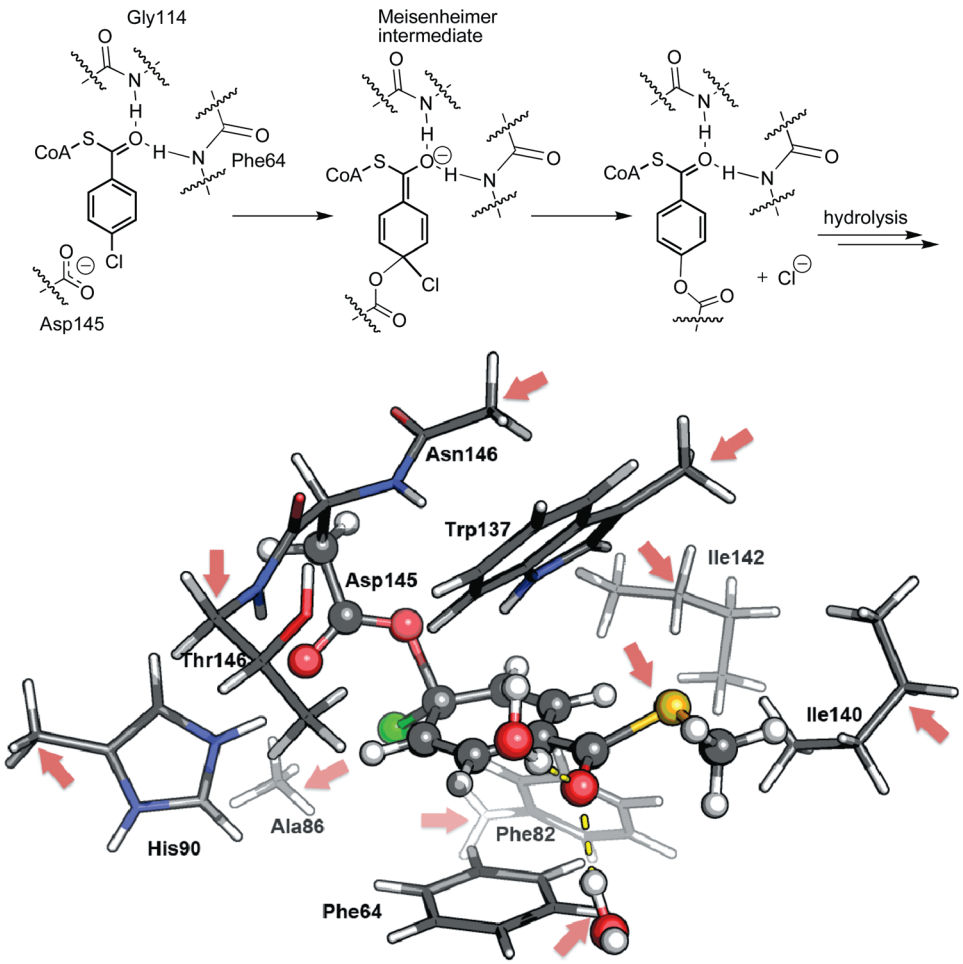

Fig. 6 Above: aromatic nucleophilic substitution in 4-chlorobenzoyl-CoA dehalogenase mechanism. Below: simplified structure used in ONIOM calculations. The atoms included in the high-level layer are represented by a ball and stick model, and the atoms in the low-level layer by a wire model. The arrows point to atoms fixed during optimization.

the effect of the Glu232 residue on the orientation of the Asp145 carboxylic acid[63]) and does not include entropic effects. Therefore, the barrier height calculated does not correspond to k_{cat} of this enzyme. Moreover, as we calculate neither the absolute reaction barrier in solution nor the preorganization energy, we cannot estimate the rate enhancement produced by the enzyme. However, as will be detailed below, this simple model can be used to study the effect of the H-bond donors orientation if this parameter is changed in alternative geometries.

The activation energy of the structure in which the H-bond donor orientation was fixed to that present in the X-ray structure (dihedral angles of 109° and 118° measured with respect to the S substituent) was compared with the activation energy when the two water molecules were allowed to optimize their positions (with the single constraint of a 4.5 Å distance between the O atoms), and to the activation energy when the dihedral angles were fixed to 90°. As expected, when the two water molecules were free to move we obtained the transition state with the lowest energy, since this corresponds to the optimization of the largest number of parameters. In this case, the optimal dihedral angles were 42° and 176° and the structure was 2.6 and 1.7 kcal mol^{-1} more stable than the two cases with fixed dihedral angles. However, after calculating the energy of the reactant states, the activation barrier in this case was 2.3 kcal mol^{-1} higher than in the case where the dihedral angle was fixed to 90° and 2.4 kcal mol^{-1} higher than fixing the dihedral angles to the values in the crystal structure, since the energies of the reactant states for these structures are 4.9 and 4.1 kcal mol^{-1} higher.

These results confirm our previous observation: the reduction in the energy barrier when the H-bond donor dihedral angles are fixed to values near 90° is not a consequence of finding the most stable TS, but rather of avoiding the most stable RS. To further test this, we compared the energy corresponding to the reactants in the three cases. The dihedral angle of the water molecules also affects the interactions of these water molecules with the other amino acids in the theozyme. This effect can be easily calculated by subtracting from the RS energy the single point energy obtained after removing the atoms corresponding to the substrate. To facilitate the calculations the Asp145 residue was included in the low level ONIOM layer. With these approximations, the energies of the reactant states were 4.4 kcal mol^{-1} (dihedral angle fixed to 90°) and 3.7 kcal mol^{-1} (dihedral angle fixed to the values in the crystal structure) higher than the relaxed reactant state.

The use of gas phase calculations to rank competing reaction pathways

Even if it were possible to find simplified models of oxyanion hole active sites which do not require the use of arbitrary constraints, the results would be meaningless if gas phase calculations cannot rank the catalytic activity of the competing active site structures. In his landmark analysis of enzyme catalysis (Fig. 7), Warshel[1,27] divides the water and the enzyme active site stabilization of forming charges into two components: the interaction with the water molecule dipoles ($\Delta G_{Q\mu}$) and the reorganization energy as the dipoles move with respect to each other ($\Delta G_{\mu\mu}$). The first of these has rather similar values in water (w)

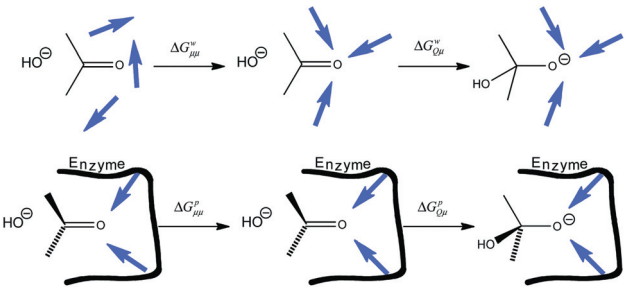

Fig. 7 Adapted from ref. 27, Fig. 4. The orientation of the carbonyl with respect to the dipole interactions is shown by means of hash&-wedge bonds.

and in enzyme active sites (p for protein), because enzymes and water are both good at stabilizing charges, so:

$$\Delta G^{w}_{Q\mu} \approx \Delta G^{p}_{Q\mu}$$

The reorganization energy, however, is very different in water and in an enzyme active site: the enzyme has preorganized the dipoles; water has to reorganize to allow the dipoles to point in suitable directions. As a result, the reorganization energy is much larger for water than for an enzyme:

$$\Delta G^{w}_{\mu\mu} \gg \Delta G^{p}_{\mu\mu}$$

The reduction in energy barrier from reactions in water to reactions catalyzed by enzymes, therefore, depends principally on the difference in reorganization energy ($\Delta G_{\mu\mu}$) between water and enzyme active sites. Calculations that do not include the reorganization energy do not provide a complete picture of enzyme reactivity.

Our analysis of oxyanion holes addresses the question of why the preferred orientation of hydrogen bonds in the enzyme active site is not the flat structure expected. Our calculations, therefore, focus on the comparison of the catalytic activity provided by alternative active site geometries with hydrogen bonds in the traditional flat or *grand jeté* orientation. The calculations do not attempt to calculate the difference between enzyme catalysis and catalysis by water. The analysis of the absolute catalytic activities of every possible geometry requires the comparison of the interaction and preorganization terms in the active site with these terms in water. However, as there is only one reaction in water, the same values of $\Delta G^{w}_{\mu\mu}$ and $\Delta G^{w}_{Q\mu}$ should be used for both flat and *grand jeté* arrangements. When comparing the catalytic activities the water term cancels. In addition, for these two very similar enzyme mediated reactions, the reorganization energy ($\Delta G^{p}_{\mu\mu}$) is unlikely to be substantially different, particularly as it is always small for enzymes:[1,8]

$$\Delta G^{w}_{\mu\mu} \gg \Delta G^{p}_{\mu\mu}$$

The interaction of the dipoles with the forming charge ($\Delta G^{p}_{Q\mu}$), however, is different for the two hydrogen bond orientations. We compare the electronic interaction energies of alternative arrangements between each simplified enzyme active site and the forming charges in the transition state $\Delta E^{p}_{Q\mu}$. This

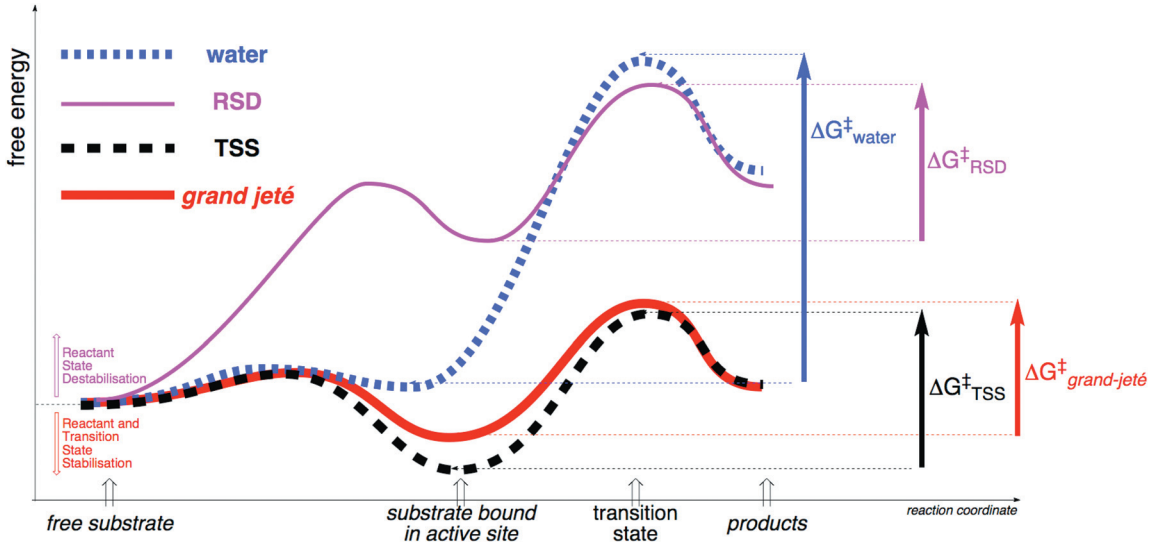

Fig. 8 Comparison between water catalyzed reactions (blue dashed line); reactant state destabilization (RSD, magenta line); transition state stabilization (TSS, black dashed line); the *grand jeté* transition state (red line). The barrier for the *grand jeté* ($\Delta G^{\ddagger}_{grand\ jeté}$) is the smallest. The free energy corresponds to just that part of the substrate which is reacting.[72] Favorable binding interactions with the rest of the substrate make the overall binding energy favorable in all cases.

corresponds to the comparison of $\Delta G^{p}_{Q\mu}$ terms with the reasonable assumption that entropic terms and long range interactions are similar for both structures.

Our calculations are reminiscent of Houk's theozymes,[43] defined as "*an array of functional groups in a geometry predicted by theory to provide transition state stabilization*", and that have been widely used in the search for artificial enzymes and catalysts.[44,45,64–68]. The arguments used for our gas phase calculations justify the optimization procedure for obtaining theozymes (see ESI†), but, unlike our gas phase calculations, theozyme calculations neglect the interaction energy with the reactant state:

$$\Delta E^{p}_{Q\mu} = E^{p}_{Q\mu}(\mathrm{TS}) - E^{p}_{Q\mu}(\mathrm{RS}) \approx E^{p}_{Q\mu}$$

This is reasonable provided its value is small compared with the interaction energy with transition state, and this approximation usually gives good results. For example, Houk has observed that the theozymes obtained for a set of reactions match the real structure of corresponding enzymes bound to inhibitors[45] (RMSD for "catalytic atoms" of only 0.67 Å, compared to 1.43 Å between the optimized structures of the active site and the enzyme–substrate structure). In addition, in structures similar to oxyanion hole active sites, Anslyn *et al.*[69,70] and more recently Pápai *et al.*[71] have found that the *grand jeté* arrangement is more effective stabilizing enolate intermediates in enzymes. However, in oxyanion holes, the arrangement of hydrogen bonds which gives maximum transition state stabilization corresponds to a H-bond dihedral angle value near 0° and not the 90° observed (Fig. 1). In this case it is not reasonable to assume that the flat and the *grand jeté* arrangements interact in the same way with the substrate of the reaction: the flat orientation that best stabilizes the transition state also best stabilizes the reactant. Twisting the hydrogen bonds reduces the transition state stabilization but reduces the reactant stabilization to a greater extent, reducing the overall energy barrier.

Since the explanation of the dihedral angle distribution of H-bond donors in oxyanion holes implies a sub-optimal arrangement for the reactant state, it could be suggested that it corresponds to reactant state destabilization (RSD; Fig. 8, magenta line). This is not the case. RSD implies that the enzyme destabilizes the reacting fragments of the substrate reducing the energy barrier to the transition state. In order to form the Michaelis complex, the non-reactive part of the substrate has to establish stabilizing interactions with the enzyme that are not affected as the reaction proceeds. In the case of oxyanion holes, H-bonds in the oxyanion hole stabilize the reacting part of the ground state. For example, in 4-chlorobenzoyl-CoA dehalogenase, the comparison of the association constants of the natural substrate and 4-chlorobenzyl-CoA, which lacks the H-bond acceptor carbonyl oxygen, reveals that oxyanion hole H-bonding contributes around 3 kcal mol^{-1} ground state stabilization[60] (this may be an overestimate since the more flexible inhibitor is conformationally restricted in the enzyme active site compared to the natural substrate). In this enzyme, the H-bond dihedral angles are far from the 0° optimum. The main factor contributing to this stabilization is the smaller preorganization energy in the enzyme than in bulk water, which compensates for the non-optimal arrangement of the H-bond donors in the Michaelis complex.

Conclusions

1. The hydrogen bond arrangement in oxyanion holes does not correspond to maximum transition state stabilization. Our analysis of oxyanion hole stability is not reaction state destabilization (RSD). However, the energy of the reaction state has to be considered in order to understand the reason for the arrangement of hydrogen bonds in oxyanion holes.

2. Using subtilisin as an example, we have shown that the dihedral angle of the H-bond donors is conserved during MD simulations. The addition of constraints to these H-bonds resulted in their elongation to a point in which they can be considered to be broken. Therefore, the dihedral angle distribution that we observed in our previous work is not lost in the enzymatic reaction transition state as a consequence of an induced fit.

3. Gas phase models of the enzyme active site can be used, within some approximations, to rank alternative geometries of the active site geometry. The use of this kind of simplified model in many theozyme applications, and in our previous paper, is reasonable. Theozyme calculations cannot be used to calculate of the overall catalytic activity of an enzyme because they do not calculate the preorganization energy in water. However, they are suitable for the less ambitious goal of estimating the contribution of particular geometric features to catalysis. Recently Pápai *et al.*[71] pointed out: "*If the positions are important, the only computational meaningful way to understand their effects is to constrain the positions of the Hydrogen Bond donors in the calculations*". As our MD simulations reveal, constraining the hydrogen bond donors positions in the enzyme to adjust their orientation results in breaking the H-bonds. As a result, calculations on simplified models are the only practical way of studying these contributions.

4. The use of constraints in calculations of simplified models of the enzyme active site is necessary in order to avoid collapsing of the structure during the optimizations. These constraints reproduce the rigidity of the active site within the protein framework, but in some cases they might be excessive and arbitrary. To test whether this could have affected the results in our previous work, we have performed new calculations with a minimum number of constraints: only the atoms from the backbone of the enzyme were frozen. The results are very similar to those we observed before.

5. The most important factor for explaining enzyme catalytic activity is transition state stabilization (TSS), and this has been demonstrated by both theoretical and experimental studies. This does not mean, however, that other effects make no contribution to catalysis. These other effects (for example, H-tunneling or dynamic effects) have been accepted without breaking any paradigm because of their limited scope and magnitude. The same is true of our observations: we have found an effect in oxyanion hole enzymes with an impact of around 2 kcal mol^{-1}, which is a small fraction of the overall catalytic activity of these enzymes. This is an effect that can readily be understood and should be introduced in the design of enzyme inhibitors and of enzyme-inspired organocatalysts.

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