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# Enzyme Catalysis by Hydrogen Bonds: The Balance between Transition State Binding and Substrate Binding in Oxyanion Holes

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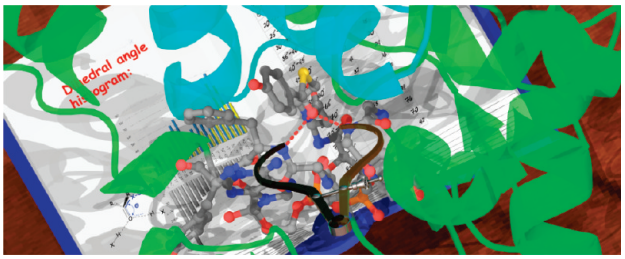


Oxyanion holes stabilize oxygen anions in transition states. Data have been gathered both from enzyme structures and from corresponding structures from the Cambridge Crystallographic Database. The two data sets show a striking contrast. The small molecule interactions in the Cambridge database optimize hydrogen bonding. The enzyme active sites do not. Analyzing the data with the help of DFT calculations on theozyme-like models, we conclude that enzymes have not optimized binding to the transition state structures in reaction pathways involving oxyanion holes, because the best binding arrangement for the anions also optimizes binding for the starting materials of the reactions. Instead, enzymes arrange the hydrogen bonds so that the oxyanions are stabilized reasonably, but suboptimally, in order to avoid overstabilization of the ground state.

## Introduction

The catalytic activity of enzymes is commonly attributed to their ability to stabilize transition states. This idea has its origin in Pauling's suggestion that the catalytic activity of enzymes is a consequence of the stabilization of the activated complex (transition state) and on Polanyi's earlier work on nonenzymic catalysis.[1] This implies that the extremely high catalytic proficiency of enzymes[2,3] is a measure of the association constant between the enzyme and the transition state.[4] Recent computer simulations[5−8] have also shown that enzyme active sites are complementary to transition states. The recent designs for artificial enzymes by Houk et al.[9,10] have aimed "to create an idealized active site with protein functional groups positioned so as to maximize transition state stabilization". In these studies, the design of artificial protein enzymes consists of the construction of theozymes, which are defined as "an array of functional groups in a

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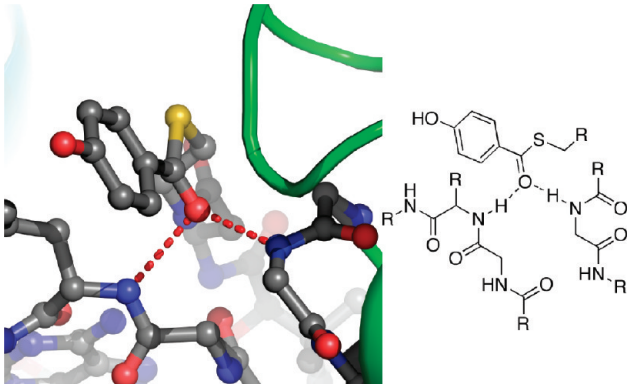

**FIGURE 1.** Structure of the oxyanion hole in 4-chlorobenzoyl Co-A dehalogenase (PDB ID: 1NZY) and a schematic representation.

geometry predicted by theory to provide transition state stabilization",[11,12] followed by the application of inverse folding tools to determine an appropriate amino acid sequence. Theozymes are useful tools in understanding the origin of the catalytic activity of enzymes, since their simplicity makes possible the straightforward interpretation of the results. Similar ideas have been used in the rational design of new organocatalysts.[13−15] Other more complicated and expensive techniques,[16] such as potential of mean force, QM/MM, or empirical valence bond simulations, are also available to interpret enzyme binding to transition states.

In this paper, we examine the applicability of these ideas to the structures known as oxyanion holes in enzymes, comparing the maximum stabilization of the transition state with their consensus geometries. Oxyanion holes consist of two or three hydrogen bond donors oriented toward a central oxygen atom in the substrate. Figure 1 shows an example with two hydrogen bond donors, both of which are amides. As a nucleophile reacts with the thioester in the substrate, electron density around this oxygen atom increases and so does its capacity to accept hydrogen bonds. The stabilization of the oxyanion by the hydrogen bonds in the transition state should contribute to the catalytic activity. Similar structures exist in small molecules, and we have used the small molecule crystal structures to investigate the geometry and hydrogen bond pattern of carbonyl compounds in oxyanion holes in these systems.

In an important set of organocatalysts, thiourea-based structures,[17,18] transition state stabilization is provided by a set of hydrogen bonds, usually two, that are able to establish strong interactions with the transition state. For these catalysts an analogy with enzyme oxyanion holes is often invoked. A better understanding of biological systems with oxyanion holes, therefore, could also be of great value in the design of organocatalysts.

## Methods and Results

Oxyanion holes were gathered from crystal structures in the PDB[4] of peptidases bound to peptide chains and of proteins bound to ligands containing a carbonyl group.[19] The structures in which at least two possible hydrogen bond donors are close to a carbonyl oxygen were identified. The search gave us 1587 structures. Further refinement was necessary because it is likely that some of these structures do not have a catalytic function. We selected 441 enzymes for which an oxyanion hole should benefit the reaction using the EC numbers of the structures and the MACIE database,[5] which contains information about enzyme reaction mechanisms (see the Supporting Information for the complete list of EC numbers accepted in this set). The Catalytic Site Atlas (CSA)[6] was used to further refine the set, by omitting those enzymes for which none of the residues in the oxyanion hole is an active residue in the mechanism. This left just 252 enzymes. A frequency plot of the distance between hydrogen bond donors ($d_1$) shows a bimodal distribution with peaks at 2.8−2.9 and 4.3−4.6 Å for the 1587 structure unrefined set (Figure 2a). For the EC-filtered 441 structures, the distribution around 4.3−4.6 Å was greatly enriched (Figure 2b); after further filtering with the CSA (252 structures set, Figure 2c) nearly all the structures fell in this region. In the former set, we identified 12 PDB entries with oxyanion holes containing three hydrogen bonds instead of just two. There were only three such three-pronged structures in the CSA filtered set. Even though the study of the geometry of the three-pronged oxyanion holes would be interesting, this set is rather too small for the extraction of statistical information, and so we have excluded these structures from our study. In addition, since the CSA does not cover all PDB entries, this double-filtered set is probably too restrictive, and so the analyses of structural parameters were carried out with use of both the 249 double-filtered structures and a larger set (310 structures, see the Supporting Information for the complete list) comprising all structures from the EC-filtered set for which the distance between the main hydrogen bond donors ($d_1$) was greater than 3.0 Å. The parameters obtained with both sets are similar.

To further investigate the structure of the oxyanion holes, the hydrogen bond patterns of carbonyl compounds, extracted from small molecule crystal structures, were also analyzed. The data were extracted from the Cambridge Structural Database (CSD),[20] searching for carbonyl compounds with one or two hydrogen bond donors (an O−H distance of between 1.5 and 2.5 Å): 43570 single hydrogen bonded and 4618 double hydrogen bonded structures were obtained.

In Figure 3, the frequency plots for six different structural parameters are shown: two distances [(a) $d_1$ is the distance between the X atoms of the hydrogen bond donors (as Figure 2) and (b) $d_2$ is the distance from the X atom of the hydrogen bond donor to the carbonyl oxygen], two angles [(c) $a_1$ is the C═O···X angle and (d) $a_2$ is the X···O···X

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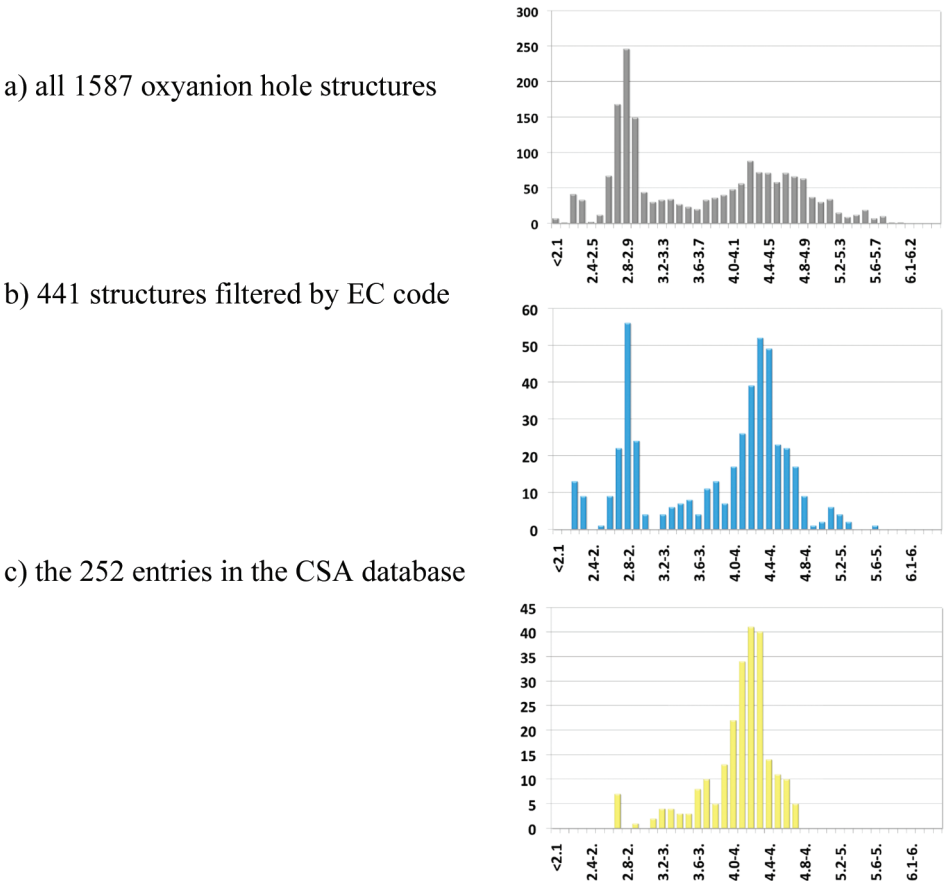


**FIGURE 2.** Frequency plots for distance between hydrogen bond donors for (a) all 1587 possible oxyanion hole structures, (b) for 441 structures filtered with appropriate EC codes, and (c) for the subset of 252 entries contained in the CSA database (see text for details).

angle], and two dihedral angles [(e) Φ is the X···O=C···X dihedral angle and (f) Θ is the X···O=C−R dihedral angle].

Yellow bars represent the data obtained from the 249 double filtered two hydrogen-bond oxyanion hole structures, for which EC numbers were compatible with hydrogen bond catalysis and at least one residue is listed in the CSA database. Blue bars correspond to the entries with appropriate EC numbers and for which distance between the hydrogen bond donors is higher than 3 Å. Data extracted from small molecule crystal structures are represented by the gray bars, which show similar trends to the distribution observed in previous analyses of hydrogen bond geometries.[21−25] This distribution corresponds to the more stable arrangement of hydrogen bonds with carbonyl groups. In contrast, the arrangement in the oxyanion hole of enzymes is a consequence of the evolution of enzymes toward the higher catalytic activities. Therefore, differences between both distributions might reveal geometric features involved directly in the catalysis.

The comparisons show similar distributions (the means and modes differ only slightly, and the overall shapes of the plots are not dramatically different) in the small organic molecules and in the oxyanion hole structures in all frequency plots except one: the X···O=C—R dihedral angle, Θ.

The distance between the hydrogen bond donors ($d_1$, Figure 3a) is the only one of the parameters that does not depend on the position of the substrate. This parameter will reflect the transition state orientation if the induced fit principle[26] does not have a major effect for these features of the active site of the enzymes in our data set. The position of some amino acid residues in an enzyme structure can change to adopt a geometry compatible with the transition state structure. For example, Gao has observed that in a cysteine protease, a glutamine residue side chain that participates in the oxyanion hole is not oriented toward the carbonyl oxygen until the reaction approaches the transition state.[27] This reorganization could be of great relevance in enzymes catalyzing multistep reactions.[28] Nevertheless, hydrogen bond donors in oxyanion holes are usually NH from the backbone, which constitutes a quite rigid region of the protein. Changes in their positions to fit the reaction transition state are not likely to occur very often in such cases, so the values in Figure 3 should correspond to those present in

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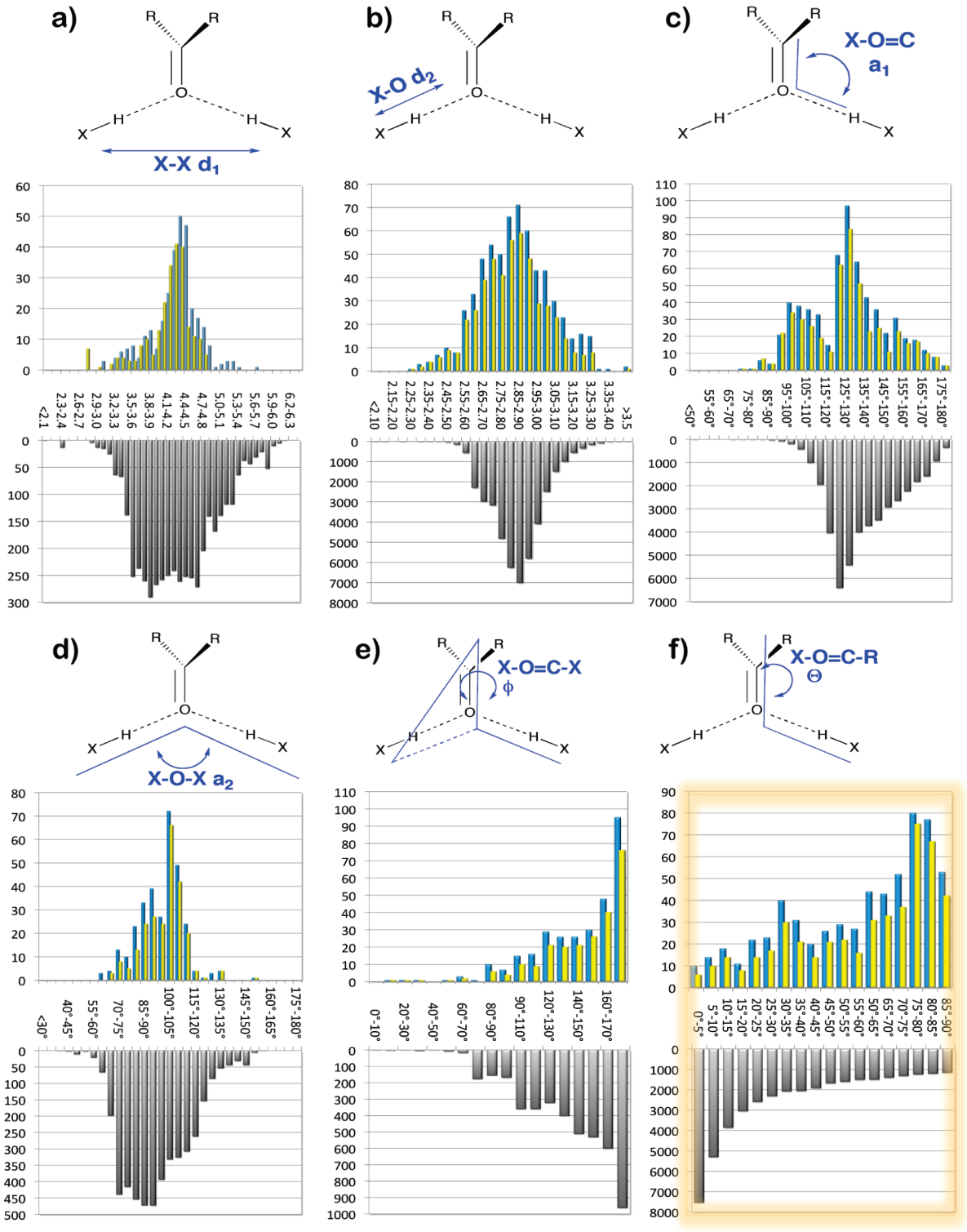


**FIGURE 3.** Frequency plots of the structural parameters in oxyanion holes. Yellow and blue bars show enzyme data; gray bars show CSD data. Distances are in angstroms and angles in degrees. Note that the patterns for CSD data reflect the enzyme data in all cases except panel f.

the reaction transition state. This suggestion is supported by the observation of a narrow distribution for $d_1$ in the oxyanion hole structures, whereas the distribution of the data extracted from the CSD shows a broader spread, with more examples at shorter distances. This difference is probably due to double hydrogen bond donors such as urea and thiourea groups. Many organocatalysts that are expected to act as oxyanion hole mimics fall into this category.[29−40]

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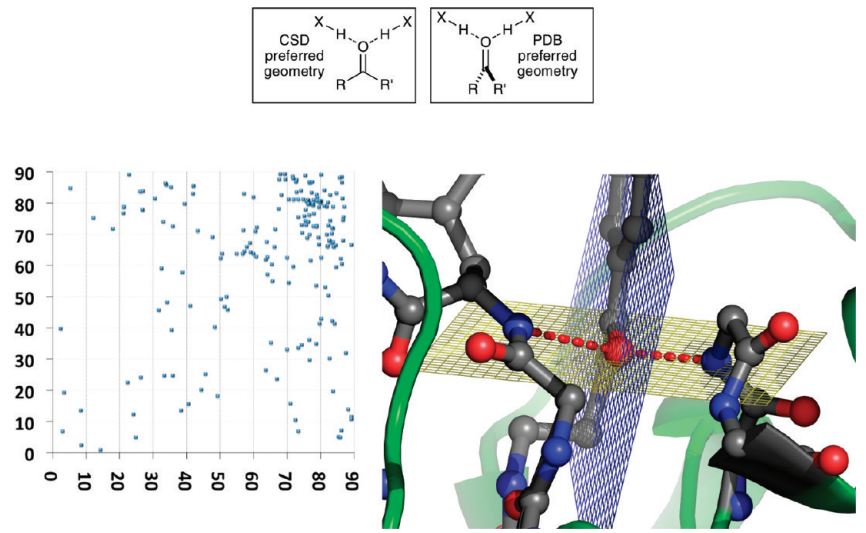


**FIGURE 4.** Scatter plot of the pairs of dihedral angles Θ (X···O═C—R) for each oxyanion hole. On the right, a representation of the dihedral angle for the hydrogen bond donor placed on the right in the oxyanion hole of 4-chlorobenzoyl Co-A dehalogenase bonded to 4-hydroxybenzoyl Coenzyme A (PDB ID 1NZY; see Figure 1).

The distance between hydrogen bond donor and acceptor ($d_2$, Figure 3b), the angles formed between hydrogen bond donor, acceptor, and α carbon atom ($a_1$, Figure 3c) or hydrogen bond donor, oxygen acceptor, and hydrogen bond donor ($a_2$, Figure 3d), and the dihedral angle between the two hydrogen bond donors around the carbonyl bond (Φ, Figure 3e) show only small differences. It is not possible to discard that these differences are important in catalysis, but their magnitude is so small that they could also be the consequence of the heterogeneity of CSD data employed.

Striking differences, however, were observed between the two sets for the dihedral angle between the hydrogen bond donors and the carbonyl, Θ, where opposite trends are observed (Figure 3f). The CSD results are consistent with (and partly responsible for) the classical view of the participation of the coplanar lone electron pairs from the carbonyl oxygen in the hydrogen bond formation,[23,25] and computational analysis[25] suggests that this distribution follows the energetic preferences of the system. Nevertheless, for oxyanion holes, the hydrogen bond donors are preferentially placed in a position almost perpendicular to the plane of the carbonyl group. An analysis of tetrahedral oxyanions in the CSD, including phosphates, sulfates, and chlorates, found a only very weak preference for the hydrogen bond donor to eclipse an oxygen atom.[21]

Figure 4 shows the distribution of the dihedral angles (Θ) for each pair of hydrogen bond donors of an oxyanion hole as a scatter plot. In agreement with the frequency plots, points accumulate in a region in which both hydrogen bonds show angles close to 90° and there are very few points in the lower left quadrant where both dihedral angles are less than 45°. Figure 4 also includes a graphical representation of the dihedral angles for the oxyanion hole in 4-chlorobenzoyl Co-A dehalogenase (Figure 1), for which Θ = 90° for both hydrogen bonds.

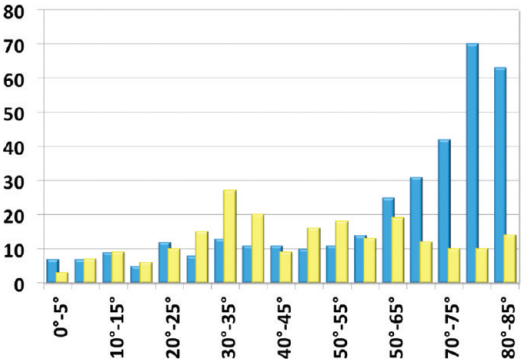


**FIGURE 5.** The blue bars show the distribution of the dihedral angle Θ (X···O═C—R) when covalent enzyme−ligand complexes are excluded, and correspond to Figure 3f. The yellow bars show the data for the covalent enzyme−ligand complexes only.

In some of the structures analyzed, the substrate is bound covalently to the enzyme and these covalent bonds might force the adoption of the observed high values of the dihedral angle Θ. Nevertheless, when the data corresponding to covalently bound ligands (116 pdb entries) were omitted from the distribution, the same trend was observed for the dihedral angle (Figure 5, blue bars, is similar to Figure 3f). This indicates that the observed distribution is not an artifact from the inclusion of data corresponding to covalent complexes. When the Θ angles corresponding only to these constrained structures are represented (Figure 5, yellow bars), a completely different distribution is obtained, further demonstrating that this possible constraint does not favor values of Θ close to 90°.

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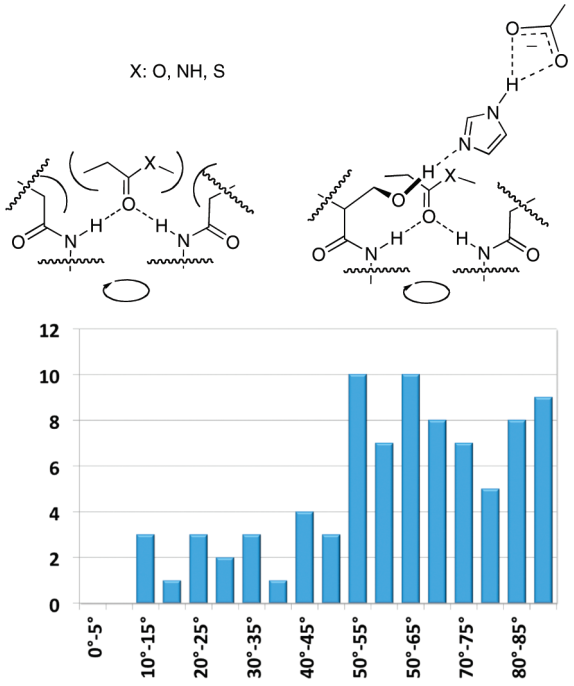


**FIGURE 6.** Schematic representation of steric effects (top left) and strain effects in serine hydrolases (top right). The frequency plots (below) are Θ values for serine and cysteine hydrolases covalently bound to a tetrahedral intermediate.

A possible factor in the choice of the dihedral angle in the enzyme complexes is the steric interactions between the ligand and the enzyme backbone (Figure 6). However, similar steric interactions should be present in the small organic molecule crystal structures, but in this set the preference for higher dihedral angles is not present and so the backbone steric interactions are unlikely to explain the difference between the PDB and CSD structures. For serine protease enzymes, the oxyanion hole includes a serine residue to which hydroxyl group reacts with the substrate carbonyl leading to the tetrahedral intermediate. The attack of this hydroxy group on the carbonyl imposes a distance constraint between both atoms, which should be close in the Michaelis complex. This distance requirement can induce a tilt of the substrate, increasing the dihedral angle. Similar constraints must also be present in serine protease active sites covalently bound to the tetrahedral intermediate, so an investigation of these structures should show if the distribution of the dihedral angle Θ is a consequence of this effect. Dihedral angles for the serine protease structures (42 entries, Figure 6) show a prevalence of Θ values ranging from 50° to 90°, but after comparing Figures 6 and 5 or 3e, the preference for the perpendicular arrangement is not quiet as clear as that for the remaining oxyanion holes (see the Supporting Information for details of how this angle is measured). The constrains introduced by the covalent bond between the enzyme and the substrate are probably responsible for the preference of angles bigger than 50°, but, over this threshold, do not impose any preference for higher angles. The prevalence of angles higher than 80° in Figure 5 (or Figure 3e) cannot be explained considering only a proximity requirement between the serine oxygen and the carbonyl group (moreover if it is considered that the shortest distance between the two groups is observed in the covalent bond complexes). Therefore, the strain requirement in serine proteases is compatible with the perpendicular arrangement for the hydrogen bonds, but it is probably not responsible for it; if that were the case, no preference for dihedral angles over 80° will be observed and the trends in Figure 5 or 3e would be similar to that in Figure 6.

Unlike other geometrical features in which the ligand atoms are involved, small displacements of the substrate as the reaction proceeds toward the transition state are not likely to make significant changes to the angle Θ. This would require the rotation of the substrate in the active site of the enzyme, and this rotation is hindered by interactions with other groups in the catalytic site. In most of the oxyanion holes studied, H-bond donors are protein backbone NH groups. Because the backbone constitutes a fairly rigid region of the enzyme, the dihedral angles are unlikely to change as the result of an induced fit. Since neither steric effects nor strain effects in serine hydrolases can give a definitive explanation for the different Θ angle distribution, it is very likely that the perpendicular arrangement of the hydrogen bond donors provides some advantage for catalysis.

Thiourea-based organocatalysts show high catalytic activities and selectivities and their design is inspired in the geometry of complexes with carbonyl substrates. Our results suggest that they might not be excellent oxyanion hole mimics. Guanidinium ions from arginine side chains (which are structurally similar to urea groups) are not found in the natural oxyanion holes in our survey. The same observation can be made for TADDOL[41−48] and BINOL[43,49,50] groups, which are also used in many organocatalysts; studies on the reaction mechanisms show that, for these catalysts, only a single hydrogen bond with the transition state is established.[41,45,46,51] So far, there have been few applications of scaffolds such as Sigman's amine functionalized oxazolines,[52,53] Kelly's biphenylenol,[54] or Morán's diamidoxanthones,[15,55,56] for which the distance between the hydrogen bond donors is more like that found in our survey.

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To investigate the catalytic effect of the hydrogen bond pattern, we have performed theozyme-like calculations on a set of reactions that could be catalyzed by an oxyanion hole. Theozymes have two important advantages over other theoretical methods for understanding enzymatic catalytic effects: first, unlike complex molecular dynamic simulations, the effect of individual groups in the overall catalytic effect can be easily determined; second, theozymes can be calculated for reactions with no known enzyme. This feature, that has recently allowed the de novo design of artificial enzymes,[9,10] is used in this work to calculate the optimal arrangements of stabilizing groups around simplified substrates in order to minimize the contribution of steric and strain effects.

Our first calculations (full details in the Supporting Information) investigated the hydrogen bonding of two water molecules to formaldehyde, to phenolate, and to methoxide. As we anticipated, the formaldehyde preferred to form planar hydrogen bonds, and the methoxide showed no significant preference for any particular hydrogen bond dihedral angle. This agrees with Hay's analysis of CSD data.[21] Phenolate oxyanions, like formaldehyde, show a preference for planar hydrogen bonds, presumably due to $\pi$-conjugation to the aromatic ring.

We extended the calculations to examine at the transition state the addition to a carbonyl, by constructing models for the first mechanistic step of serine esterase (X = O in Figure 7a), peptidase (X = NH), thioesterase (X = S), and a hypothetical enzyme catalyzing the hemiacetal formation (X = $CH_2$). The oxyanion hole was substituted by two water molecules, which have similar hydrogen bond donor properties to the hydroxyl groups in serine and threonine and also to NH groups from a protein backbone,[57] but that minimize the steric effect imposed by other possible models such as acetamide or methylacetamide. Acetic acid and indole molecules were included as models for aspartic or glutamic and histidine residues in the catalytic triad. Previous theozyme calculations on enzymes containing catalytic triads support this choice, since systems of this type have led to accurate results within a reasonable computational time.[11,28] Unlike these previous studies, the model for serine is an intramolecular OH group. This approximation was used to minimize steric contacts and to release any constraint imposed by the double role of the serine in the reaction mechanism. The (carbonyl)O−αC−(model Ser)O−(model His)N dihedral angle was fixed to 150° (Figure 7a). Calculations comprised a relaxed two-dimensional scan of the αC−(model Ser)O distance of the forming bond and of Θ (X···O═C—C), using DFT. Geometry optimization were carried out by B3LYP[58] functional combined with 6-31G(d,p)[59−61] basis set, and single point energy evaluations using MPWB1K[62−65]

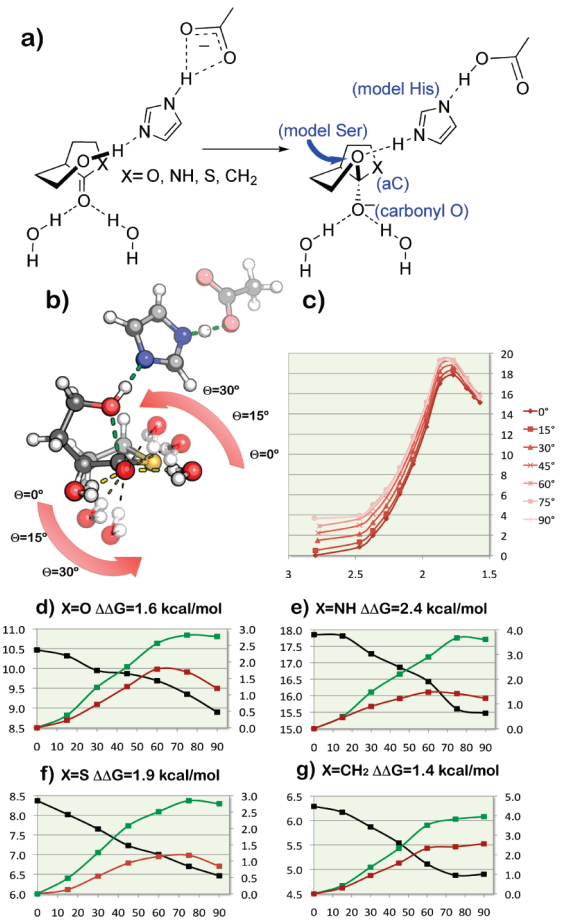


**FIGURE 7.** (a) Model enzymatic reaction studied; (b) dihedral angles that are changed in the two-dimensional scan; (c) reaction profile for X = NH. The four graphs d, e, f, and g show the barrier height (black, scale on the left), transition state, and substrate loss of stabilization (red and green, respectively, scale on the right) as a function of the dihedral angle for X = O (d), NH (e), S (f), and $CH_2$ (g). Energies are expressed in kilocalories/mole, and distances in angstroms.

functional with 6-311++G** basis set[59,66,67] were performed on the resulting structures. During the optimizations, other geometrical variables (see the Supporting Information) were constrained to the most common values obtained in previous analyses of oxyanion hole structures. The inclusion of the constraints allows for the rigidity of the enzyme active site. If these constraints were not used, the optimizations often lead to structures in which water molecules interact with other heteroatoms in the system such as the X (O, S, or N) atom in the substrate, the hydroxy group of the serine model, the indole, or the carboxylate atoms. The constraints in the model are reasonable, but not the only possible constraints that could be used to investigate this process. Our constraints (detailed in the Supporting Information) produced a reaction profile with a transition state close to, but distinct from, the product $sp^3$ anion.

These calculations show, as expected, that the ground state of the reaction is lowest in energy when the hydrogen bonds are in the plane of the carbonyl (Θ = 0°). As the reaction

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proceeds toward the transition state, this remains the most effective conformation, and becomes the arrangement that best stabilizes the transition state ($\Theta = 0°$). Surprisingly, therefore, the enzymes are not optimized for transition state binding in this reaction. To explain the preference for higher dihedral angles it is necessary to consider not only the stabilization of the transition state, but also the different stabilization of the substrate of the reaction. The $\Theta = 0°$ conformation provides optimal transition state stabilization, but provides even greater substrate stabilization. As $\Theta$ increases, both substrate and transition state are stabilized progressively less well, but the substrate changes more than the transition state. The barrier height, therefore, is less at $\Theta = 90°$ than at $\Theta = 0°$ even though the transition state is less well stabilized at $\Theta = 0°$. The effect is greatest for the model peptidase (2.4 kcal/mol), for which the transition state structure loses 1.2 kcal/mol of stabilization, but the starting material loses more, a 3.6 kcal/mol loss of stabilization.

The smaller stabilization of the transition state structure when $\Theta = 90°$, which is observed for all reactions studied, is an unexpected result. As the reaction proceeds, the $sp^2$ carbonyl oxygen acquires more $sp^3$ character, which should facilitate the formation of the hydrogen bonds perpendicular to the original carbonyl plane. The three $sp^3$ hybrid orbitals should be distributed more or less evenly around the oxygen atom in the substrate. Therefore, the dihedral angle between any pair of these hybrids ($\Phi$) should be close to 120°. In the theozyme calculations, we have fixed this angle to 180° based on the distribution found for oxyanion hole complexes. It is possible that this constraint prevents the hydrogen bond donors from adopting a geometry in which maximum overlap with two of the three hybrid orbitals is obtained, while in the enzyme small displacements of the reacting ligand could lead to values of $\Phi$ closer to 120°. That would mean that the lesser stabilization of the transition state is an artifact caused by an over reduction in the degrees of freedom of the system during the DFT calculations. To test this possibility, we have repeated the geometry optimization for our model of the peptidase when the dihedral angle $\Phi$ was unconstrained and only one of the water molecules has constraint on $\Theta$ (Figure 8). This modification has a moderate effect on the geometries and energies of the transition states: for example, when $\Theta = 90°$ the optimal value for $\Phi$ was found at around 160° and the energy of this structure is only 0.25 kcal/mol smaller than that of the theozyme in which $\Phi = 180°$ was fixed. The differences were even smaller when $\Theta$ was reduced (Figure 8). For the substrate, the effects of releasing the $\Phi$ constraint were more relevant: when $\Theta$ was fixed to 90°, the optimal $\Phi$ was 137°, and this structure is 1.4 kcal/mol more stable than the analogue structure where $\Phi$ was fixed to 180°. As the oxyanion holes occur more often with values around $\Theta = 90°$ and $\Phi = 180°$, the preferred conformation corresponds to a suboptimal arrangement of the hydrogen bond donors. This suboptimal arrangement is not the most effective at stabilizing the transition state (the additional stabilization of the transition state when the $\Phi$ constraint was released, 0.25 kcal/mol, is not enough to compensate for the loss of 1.2 kcal/mol of stabilization from the $\Theta = 90°$ hydrogen bond pattern), but the overall effect is a reduction of the barrier height. Therefore, not only the use of $\Theta = 90°$ but also the choice of $\Phi = 180°$ contribute to the catalytic effect of the hydrogen bond donors, by stabilizing the substrate to a lesser extent than the transition state.

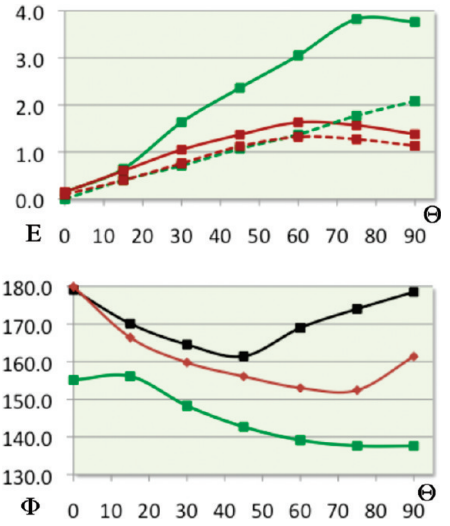


**FIGURE 8.** (a) Loss of stabilization of the transition state (red) and the reaction substrate (green) as a function of the angle $\Theta$. Continuous lines: $\Phi$ (the dihedral angle between the two water molecules) fixed to 180°. Dashed lines: $\Phi$ free to relax. (b) Optimized $\Phi$ after releasing the constraint for one water molecule for different values of $\Theta$ (green: substrate; red: transition state; black: distance O−C constrained to 1.57 Å). $\Phi$ prefers a value near 180° for both the transition state and the O−C constrained structure.

The directionality of hydrogen bonds can be related with the Laplacian of the electron density ($\nabla^2\rho$) and the position of the minima of this magnitude[23] for the nonbonded hydrogen bond acceptor. This magnitude is also related to the localized electron pairs assumed in the VSEPR model.[68] We have used the AIMPAC suite of programs[69] to locate the position of these critical points around the carbonyl oxygen atom in the theozyme model of a serine protease when no hydrogen bond donors were included. The structures were reoptimized when the distance between the nucleophile oxygen and the carbonyl C atom was fixed to 1.77 Å (corresponding to the transition state when the two water molecules were present), to 1.57 Å, and when this constraint was released so the structure can relax back to the substrate. Two maxima in $-\nabla^2\rho$ (which corresponds to two minima in $\nabla^2\rho$) were found in all cases. Contour plots of $-\nabla^2\rho$ represented in a plane perpendicular to the C=O bond and which contain these two maxima are shown in Figure 9. For each of the three structures, maximum values of $-\nabla^2\rho$ were observed for angles close to $\Theta = 0°$. The higher electron density in the O for the 1.77 and 1.57 Å structures leads to smaller differences of $-\nabla^2\rho$ with respect to the $\Theta$ dihedral angle when it is compared to the plot for the substrate structure. This illustrates why the transition state is less sensitive to the position of the hydrogen bond donors than the substrate, but that it maintains some preference for $\Theta = 0°$, as the transition state has not lost all substrate character.

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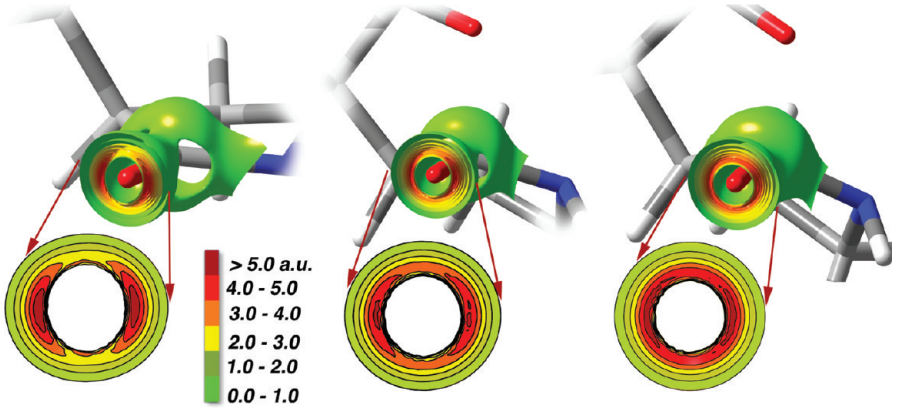


**FIGURE 9.** Isosurfaces and contour plots of $-\nabla^2\rho$ for the structure of the substrate (left) and structures optimized when the distance O−C was 1.77 (center) and 1.57 Å (right).

Herschlag et al.[70] compared charge localization to binding for different phenolates binding to the ketosteroid isomerase oxyanion hole, and found that large changes in charge localization lead to only small changes in binding. They concluded that electrostatic complementarity alone cannot account for all of the catalytic effect of the enzyme and suggested that the change from an $sp^2$ oxygen in the substrate to an $sp^3$ oxygen in the transition state might lead to a higher catalytic effect than is suggested by the weak effect on phenolate binding. Our calculations support this conclusion, and suggest that the overall catalytic effect can be enhanced by sacrificing some transition state binding in order to diminish substrate binding to a greater extent.

Warshel et al.[71] questioned Herschlag results pointing out that charge localization in phenolates also affects the solvation energy in water. The enzyme takes advantage of the preorganization[7,8] effect of the enzyme polar groups. The enzyme polar groups are already arranged to stabilize the transition state charge distribution, whereas in water the dipoles are randomly oriented. Our calculations show that catalysis with two water molecules, even if they are optimally placed to stabilize the transition state, is less effective than catalysis by the oxyanion hole, because water will not be able to stabilize the ground state less well than the transition state. The effect of preferential orientation (isolated from preorganization) is small (<2.4 kcal/mol), but still constitutes an important fraction of the overall stabilization provided by oxyanion holes. For example, site directed mutagenesis experiments on subtilisin revealed that the elimination of one of the hydrogen bond donors in the oxyanion hole reduces the catalytic contribution of the oxyanion hole by 3.2−3.4 kcal/mol.[72]

Anslyn and co-workers have observed a preference for an out-of-plane orientation of hydrogen bond donors and metal ligands with respect to the carbonyl group in enzymes involved in an enolate formation.[73,74] Their calculations show that the position of the ketone−enolate equilibrium can be shifted toward the enolate by placing the counterion at a dihedral angle of 45° with respect to the carbonyl group.[74] They attribute the effect to "backside coordination" to the breaking C−hydrogen bond, and to stabilization of the developing negative charge on the oxygen. In our study, the first effect is absent, and according to the results of the DFT calculations, the second provides less stabilization than planar coordination.

## Conclusions

Our study shows that oxyanion holes in enzymes are not optimized for transition state binding, but are arranged to minimize the barrier for the reaction. Most enzymes owe their high catalytic activity to their ability to bind the transition state of the reaction,[2,3] and so, of the different contributions to enzyme catalysis, the transition state stabilization seems the most important effect.[5] Nevertheless, the traditional explanation for the oxyanion hole activity, the higher electrostatic interactions with the transition state than with the substrate, is not fully satisfactory since enzymes show their catalytic activity in water, and water molecules should also be able to establish strong interactions with the transition state. Unlike water, enzymes can choose to orient their hydrogen bonds to stabilize the transition state slightly less well than is optimal, in order to stabilize the substrate much less well than is possible with the same number of hydrogen bonds.

Unlike other contributions to enzyme catalysis (such as tunneling or other dynamic effects, which usually have a similar quantitative impact in the overall catalytic activity[5]), this suboptimal arrangement of stabilizing groups to provide a greater reduction of the energy barrier (instead of higher transition state stabilization) can be readily introduced as a factor in the design of artificial catalysts. Therefore, just as transition state stabilization has inspired the development of artificial catalysts, we hope that this observation could lead to the design of more effective catalysts.

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**Supporting Information Available:** The procedure to find possible oxyanion holes, a list of EC codes for enzymes that have been included as possible oxyanion hole containers, a list of 310 PDB structures after filtering for Hbd1-Hbd2 distance greater than 3.0 Å and with appropriate EC codes, calculation of dihedral angle between H-bond donor, H-bond acceptor, α-carbon, and $\beta$ substituents, analysis of the dihedral angles for ligands bound covalently to the substrate, computational details, reaction profiles from two-dimensional scans, and references for Supporting Information, including a full list of authors for ref 57. This material is available free of charge via the Internet at http://pubs.acs.org.