Specificity in lipases: A computational study of transesterification of sucrose

GLORIA FUENTES,^{1,2,3} ANTHONIO BALLESTEROS,¹ AND CHANDRA S. VERMA^{2,4}

¹Departamento de Biocatálisis, Instituto de Catálisis, CSIC, Cantoblanco, 28049 Madrid, Spain

²Structural Biology Laboratory, Department of Chemistry, University of York, York YO10 5DD, United Kingdom

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Abstract

Computational conformational searches of putative transition states of the reaction of sucrose with vinyl laurate catalyzed by lipases from *Candida antarctica B* and *Thermomyces lanuginosus* have been carried out. The dielectric of the media have been varied to understand the role of protein plasticity in modulating the observed regioselective transesterification. The binding pocket of lipase from *Candida* adapts to the conformational variability of the various substates of the substrates by small, local adjustments within the binding pocket. In contrast, the more constrained pocket of the lipase from *Thermomyces* adapts by adjusting through concerted global motions between subdomains. This leads to the identification of one large pocket in *Candida* that accommodates both the sucrose and the lauroyl moieties of the transition state, whereas in *Thermomyces* the binding pocket is smaller, leading to the localization of the two moieties in two distinct pockets; this partly rationalizes the broader specificity of the former relative to the latter. Mutations have been suggested to exploit the differences towards changing the observed selectivities.

Keywords: lipases; transesterification; sucrose; specificity; computational methods

Our fundamental concepts of enzyme mechanisms are largely based on the idea of complementarity between an enzyme and the reaction transition state (Pauling 1948). Through a combination of shape and electronic properties (Goodsell and Olsen 1990; Bacon and Moulton 1992; Jones and Thornton 1996), enzymes select the appropriate substrates for a given reaction. Transition-state stabilization appears to occur through a complex balance between favorable (e.g., hydrogen bonding, electrostatic complementarity, burial of hydrophobic surfaces) and unfavorable (e.g., bond distortion, cavity formation, entropic penalties) interactions in the enzyme–substrate complex. Such conclusions have traditionally been based on a static description of protein conformation. With developments in spectroscopy, the role

of protein mobility during catalytic or functional events (Faber and Matthews 1990; Gerstein et al. 1994; Frauenfelder and McMahon 1998) started being recognized. These include regulations of ligand diffusion into and out of proteins (Johnson et al. 1979; Mulder et al. 2001), coupled motions of flexible loops or “lids” (McCammon and Harvey 1987; Gunasekaran and Nussinov 2004), and rigid-body movements (“breathing motions”) of entire domains or subunits (Gerstein et al. 1994). A large class of protein motions are now recognized as being of importance to various aspects of functionalities (Jääskeläinen et al. 1998a; Frauenfelder et al. 2001). The fine details that characterize the spatial and temporal details of such processes only became evident with developments in computer hardware and software. Computational methods now regularly complement experiments in providing unique and incisive insights into such processes (Kazlauskas 2001; Warshel 2003; Garcia-Viloca et al. 2004).

We apply computational methods in this study to investigate the links between the structural plasticity of lipases and their functions. Lipases are among the most versatile enzymes currently in use (Schmid and Verger 1998; Pandey

Reprint requests to: C.S. Verma, Bioinformatics Institute, 30 Biopolis Way, #07-01 Matrix, Singapore-138671; e-mail: chandra@bii.a-star.edu.sg; fax: +0065-6478-9047.

Present addresses: ³Bijvoet Center for Biomolecular Research, NMR Department, Utrecht University, 3584 CH, Utrecht, The Netherlands; ⁴Bioinformatics Institute, Singapore 138671.

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et al. 1999; Pleiss et al. 2000) (<http://www.led.uni-stuttgart.de>). One of their main uses is in synthesis, such as in the regioselective monoacylation of sucrose. This reaction is carried out using a lipase-catalyzed process in a medium constituted by a mixture of *tert*-amyl alcohol (a non-toxic and slightly polar solvent, where most enzymes remain active) and a polar solvent, dimethyl sulfoxide (Ferrer et al. 1999), and leads to different positional isomers. One such lipase, that from *Thermomyces lanuginosus* (TIL), displays a high regioselectivity for the 6-hydroxyl position in the acylation of sucrose (Ferrer et al. 1999); in contrast, lipase B from *Candida antarctica* (CALB) leads to a mixture of two monoesters (the 6-hydroxyl and the 6'-hydroxyl position; Woudenberg et al. 1996). Experiments comparing the transesterification reaction of sucrose with vinyl laurate for both enzymes under identical conditions have been reported. It was found that (under conditions involving a mixture of solvents of 2-methyl 2-butanol:DMSO (4:1 v/v)) TIL was the most efficient enzyme for this reaction—with a sucrose to monolaurate conversion of 51%. On the other hand, CALB gave rise to a sucrose conversion of 45%, which consisted of the two major monoesters (see above) in approximately an equimolar ratio (Ferrer et al. 1999); the speed and ratios of product formation depended on the amount of cosolvent DMSO. So what is the origin of these differences? Although we cannot yet examine the influence of the cosolvent directly (some progress has recently been made in this difficult area; see Elcock 2003), we can approach the problem using simple computational and structural models. The main advantage that computational techniques have over experimental ones is that structural computational models can be built to explore how only one or a few of the several conformations/reaction-coordinates possible are observed experimentally. Structurally, both enzymes (Fig. 1A,B) are characterized by similar overall folds, the same catalytic triad residues, and the oxyanion hole (and the same underlying catalytic mechanism); however, there are several significant differences in the size and shape of the binding pockets (Pleiss et al. 1998) that undoubtedly will shape the reaction coordinate and hence the specificity. For example, TIL has a helical lid region covering the active site and undergoes large-scale conformational rearrangements upon ligand binding (Brzozowski et al. 2000); in the case of CALB, no such conformational rearrangement of a potential lid has been found. It is at this level that the microscopic interactions between various conformational substates of the ligand/enzyme will be the discriminating factors between the allowed and the disallowed states. Computational structural modeling is most powerful at revealing this. Indeed, we have previously successfully reproduced the above-mentioned experimental observations of differing specificities towards the sucrose moiety and its acylation with vinyl laureate in organic mixtures. This was carried out by examining computationally the conforma-

Figure 1. Secondary structures for CALB (A) and TIL (B). The catalytic triad and ligands are shown explicitly.

tional space available to the reacting species in the active site that were subsequently analyzed with simple models of enthalpy and entropy (Fuentes et al. 2002; G. Fuentes, A. Ballesteros, and C. Verma, in prep.). Here, we extend our study to examine the processes in different media (representing different dielectric properties); our aim is to probe the extent and nature of motions and the resulting structural plasticity in each enzyme and model the extent of dynamic control exerted by the enzyme on the reaction coordinate.

Results

For each isomer, the minimum energy structures corresponding to the conformational searches performed under the three dielectric media for each enzyme were examined for conformational variability. We examined (1) the changes in interatomic distributions as measured through difference distance matrices, (2) correlations between the distributions in space of the atomic positions, and (3) the changes in the geometry (as computed through the areas and volumes) of the pockets that define the ligand binding regions.

Difference distance matrices

Large changes in the interatomic separations between the different structures, which are indicative of structural plas-

ticity, were examined. The associated residues, together with contacts (including hydrogen bonds) made with the ligands, are listed in Table 1. To have an index of the fluidity, we listed the root-mean-square deviation (rmsd) between the enzyme (corresponding to the tetrahedral intermediates) and the X-ray structures. In the rest of this section, we focus on interactions that govern functionality in CALB (Table 1) alone, because relative to CALB, TIL is characterized by too many interactions at a local level; these interactions are complex, are not conserved between the various dielectrics, and hence are deemed to be unimportant in determining functionality.

CALB ($\epsilon = 2$)

We first examined the medium with the lowest dielectric constant ($\epsilon = 2$). In the case of the 6-hydroxyl adduct (henceforth to be referred to as 6-adduct), the glucose ring (containing the carbon with the primary 1' hydroxyl group, henceforth to be referred to as the 1'-adduct) contacts Leu278. On the other hand, the fructose ring (containing the carbon that carries the primary 6'-hydroxyl) is very close to Pro280. In contrast, in the case of the 6'-hydroxyl adduct (henceforth to be referred to as 6'-adduct), the only repulsion found was between the glucose ring and Pro280. Because of these interactions, in the case of the 6-adduct, the amino acid residues move to avoid the repulsions, whereas in the 6'- and 1'-adducts, they remain close to their initial, experimentally observed positions; this is further supported by the smaller rmsd values of the latter (Table 1; Fig. 2A,B). Another amino acid residue that makes contacts is Ala281. The 6'-adduct is close to its backbone nitrogen, and hence

undergoes a slight repulsion, as is evidenced by a relatively small motion (Table 1). Overall, motions of the helix (containing Pro280-Ala281) dominate the modulation of ligand conformations (Fig. 2C).

CALB ($\epsilon = 9$)

When the medium changes to $\epsilon = 9$, in contrast to the motions seen at the top of the cleft above, residues at the bottom of the cleft (Fig. 2C), namely Gly41 and Gln46, are implicated, particularly in the case of the 6- and 6'-adducts. The carbonyl carbon in Gly41 is in close contact with the oxygen in the 1'-hydroxyl group in the 6-adduct, which in turn makes a hydrogen bond with the backbone nitrogen; together this results in a hinged motion of Gly41 into the protein. At the same time, Glu46 is pushed by a repulsive interaction with oxygen O4'. In the 6'-adduct, Glu46 undergoes a conformational change leading to the formation of a hydrogen bond between its carbonyl and the hydroxyl O₃H₃ (Fig. 3A). On the other end of the pocket, the acyl chains make interactions predominantly with Gln191 (Fig. 3B). Again, as in the case of $\epsilon = 2$, Pro280 and Ala281 are involved. Although there are no close contacts with Pro280, the contacts with Ala281 lead to small displacements of this region. Whereas 6'- and 1'- adducts interact with the C β , the 6'-adduct is involved in an additional hydrogen bond leading to some movement here (Fig. 3C). In general, the movement of this helix is smaller than in the $\epsilon = 2$ case.

CALB ($\epsilon = 17$)

Upon increasing the dielectric to 17, we find that the conformations and hence interactions between the amino acid residues are generally similar to the $\epsilon = 9$ situa-

Table 1. Amino acids that make important contacts in the distance matrices, with the hydrogen bonding and rmsd for each system studied in the conformational searches for CALB

	6-adduct			6'-adduct			1'-adduct		
	Close distance	hbonds	rms	Close distance	hbonds	rms	Close distance	hbonds	rms
$\epsilon = 2$									
Leu278	C' ₁ -C α (2.1 Å)		2.1			1.3			0.4
Pro280	C' ₄ -C γ (2.1 Å)		3.7	C ₆ -C γ (2.4 Å)		1.4			
Ala281				O' ₁ -N (2.6 Å)		1.5			
$\epsilon = 9$									
Gly41	O' ₁ -C (2.4 Å)	O ₇ H ₁₂ N	1.7						
Gln46	O' ₄ -C (2.1 Å)		0.9		O ₃ H ₃ O O ₄ H ₄ O	1.7			
Gln191	C ⁿ ₁₀ -N ϵ 2 (2.6 Å)		1.4	C ⁿ ₉ -N ϵ 2 (2.7 Å)		2.8	C ⁿ ₁₀ -N ϵ 2 (2.3 Å)		2.8
Ala 281				O _{pyr} -N (2.1 Å)		1.9	H' ₆ -C β (2.4 Å) O ₄ -C β (2.9 Å)		0.8
$\epsilon = 17$									
Gly41	O' ₁ -C (2.3 Å)	O ₁ H ₁ N	3.3	C ₆ -O (2.60 Å)	O ₆ H ₆ N	1.8			
Gln46	O' ₄ -C (2.1 Å)				O ₃ H ₃ N				
	O ₄ -C β (2.5 Å)		2.5		O ₄ H ₄ N	0.6			
	C ₄ -C γ 2 (2.7 Å)								
Val190	C ⁿ ₈ -C β (2.4 Å)		1.6						2.7

Figure 2. Interactions found for the different intermediates of CALB (the 6-adduct is blue; the 6'-adduct is pink; the 1'-adduct is green) for $\epsilon = 2$; along with the crystal structure of CALB (PDB entry 1lbs) shown in ball-and-stick for reference. (A) Interactions of Leu278, Ala279, and Pro280 with 6- and 6'-adducts. (B) Interactions of Leu278 and Ala279 with 6- and 1'-adducts. (C) A surface representation of CALB with the ligand bound to highlight the relative locations of some key residues and the cleft where the active site is located.

tion. Gly41 is involved in a new interaction with the 6'-adduct. The carbon C₆ of sucrose is close to the carbonyl oxygen of Gly41, leading to larger local rearrangements than those observed in the case of the other dielectric constants. The interactions of Glu46 are conserved across the three dielectrics (Fig. 4A). The increase in dielectric and the concomitant change in local plasticity brings the acyl chain carbons into close interactions with Val190 and leads to repulsions in the 6- and 1'-adducts (Fig. 4B); this situation is unique to this higher dielectric. Again, in the case of Pro280 and Ala281, the observed interactions and structural rearrangements are quite similar to those seen in the lower dielectrics (Fig. 4C).

Correlated motions

To obtain a global picture of the rearrangements that accompany the conformational plasticity of the complexes, we

examined, for each enzyme separately, the average displacements (equivalent to fluctuations) of all of the structures taken together relative to their geometrical average. From these displacements, we computed maps of the correlations between the directions of motions of the C α atoms (Fig. 5A,B). These maps reveal concerted motions of groups of atoms and additionally reveal whether two groups of atoms move away or toward each other (negatively correlated) or move in the same direction as each other (positively correlated). We were interested in those motions that are involved in facilitating ligand diffusion into and out of proteins and additionally are responsible for enabling functions (Dvorsky et al. 2002). These motions oppose each other in direction (the regions of negative correlations) and typically span binding sites, that is, refer to conformational coupling between the residues that line the sides of binding clefts (Miller and Agard 1999; Dvorsky et al. 2002).

Figure 3. Interactions found for the different intermediates of CALB (the 6-adduct is shown in blue; the 6'-adduct in pink; the 1'-adduct in green) for $\epsilon = 9$; along with the crystal structure of CALB (PDB entry 1lbs) shown in ball-and-stick for reference. (A) Interactions of Gly41 and Gln46 with 6- and 6'-adducts; (B) interactions of Gln191 with the three adducts; (C) interactions of Pro280 and Ala281 with the three adducts.

Figure 4. Interactions found for the different intermediates of CALB (the 6-adduct is in blue; the 6'-adduct is in pink; the 1'-adduct in green) for $\epsilon = 17$; along with the crystal structure of CALB (PDB entry 1lbs) in ball-and-stick for reference. (A) Interactions of Gly41 and Gln46 with 6- and 6'-adducts; (B) interactions of Val190 with the 6- and 6'-adducts; (C) interactions of Pro280 and Ala281 with 6- and 1'-adducts.

CALB

In the case of CALB (Fig. 5A), the correlations were small and widespread across the enzyme. There is some evidence of movements of domains, such as between the stretches 110–140 and 160–200 (to the left of the ligands in Fig. 1A) and also between these regions and the 300–318 stretch (to the top left of Fig. 1A). All of these regions are on the same side of the ligand binding cleft and do not seem to directly couple the residues that form the regions involved in the postulated opening/closing motions across the ligand binding region. Stronger correlations are observed more locally and are largely between the areas that are close to the acyl pocket. This arises from the fact that in contrast to the constrained alcohol pocket, the acyl pocket is generally more spacious in lipases (Otto et al. 2000) and hence deforms easily, enabling it to accommodate a highly flexible part of the ligand.

TiL

For TiL, the correlation plot (Fig. 5B) shows stretches of regions (domains) that undergo correlated motions with re-

spect to each other. A strong anticorrelation characterizes the motion of the lid/hinge ($\alpha 5$ helix) region against regions of $\beta 5$, a loop that delimits one of the sides of the active site crevice, $\alpha 1$, $\alpha 2$, $\beta 6$, $\alpha 7$, $\beta 9$, $\alpha 11$ and the loop connecting them, the carboxyl terminus (the catalytic histidine is located in this region, in a loop just before $\alpha 11$). Many residues in this domain belong to the acyl pocket. The $\alpha 1$ – $\alpha 2$ region delimits the putative sucrose-binding pocket and additionally contains Trp21, which is involved in the putative sucrose-binding pocket. The catalytic serine (Ser146) protrudes from a short loop at the carboxy-terminal end of $\alpha 6$ helix (the so-called nucleophile elbow). As can be seen from Figure 5C, these regions, although far apart in sequence, make up the binding pocket walls. Again, we see one stretch of contiguous correlation between the 200–250 region and several other regions (Fig. 5D). In particular, the motion against the 160–180 region reflects the motions of the residues of the acyl pocket relative to those of the alcohol pocket. Furthermore, residues in $\beta 6$ move relative to regions near the loop where the catalytic serine is located while the region of the catalytic serine (146) moves relative to the turn between strand $\beta 8$ and helix $\alpha 9$ (the latter contains the catalytic Asp201). This latter motion is probably coupled to accommodation of the ligand and its orientation such that the local stereochemical requirements for catalysis are satisfied.

Geometry of the lipase pockets

The plasticity of lipase pockets was further examined by calculating the distributions in their geometries (the areas and volumes) with the program CASTp (Table 2; the ligands were excluded from the calculations). The dual entries in Table 2 refer to situations where the pocket appears subdivided into two mainly as a result of the nature of the algorithms used. There appears to be no correlations between charge screening (dielectric) and the area/volumes spanned by the pockets. The pocket in CALB is never subdivided, in contrast to TiL, which has a more complex structural organization. Although this results partly from the limitations of the algorithm used in this study, it is also a consequence of the fact that the pocket in CALB is large but is smaller and more complex in TiL. Also, the TiL-ligand interactions are characterized by longer range correlations and the influence of secondary structural elements (see section above). The existence of just one pocket in CALB leads to larger areas and volumes of its binding pockets relative to those in TiL; the areas and volumes can be up to twice as large. TiL also seems to be partitioned into multiple pockets depending on the nature of the isomer; however, the 1'-adduct is associated with only one pocket, because the ligand is oriented almost perpendicular to the surface of the enzyme, pointing into the binding cavity and hence defining only one binding pocket (Fig. 6). In contrast, the 6- and

Figure 5. Contour plots of the correlations between distributions of the C α positions. Positive values are shown *above* the diagonal and negative values are shown *below* the diagonal. The contours correspond to correlations of -0.6 , -0.3 , 0.3 , and 0.6 in CALB (A) and TIL (B); the dashed lines show the location of the lid region. (C) C α -trace of TIL in blue ribbon with ligands shown in green spheres; the lid region in red is anti-correlated in motions with the regions in yellow and the black arrows depicts this motion. (D) C α -trace of TIL in blue ribbon with ligands shown in green spheres; the 200–250 subdomain region in red is anti-correlated in motion with the regions in yellow, and the black arrow depicts this motion.

Table 2. Areas and volumes calculated for the binding pockets of TIL and CALB

	TIL		CALB	
	Area ($\text{\AA}^2$)	Volume ($\text{\AA}^3$)	Area ($\text{\AA}^2$)	Volume ($\text{\AA}^3$)
$\epsilon = 2$				
6OH	768	991	794	1001
6'OH	198	229	864	1044
	513	671		
1'OH	473	642	816	1027
$\epsilon = 9$				
6OH	208	315	704	855
	395	502		
6'OH	733	900	685	855
1'OH	479	618	713	791
$\epsilon = 17$				
6OH	258	344	718	877
	320	458		
6'OH	852	1007	811	1119
1'OH	841	1019	627	858

6'-adducts are “draped” along the surface of the enzyme with the lauroyl moieties occupying the same pocket in all 3 cases (irrespective of the dielectric) and the two sugar rings occupying different troughs on the surface; this leads to the formation of multiple pockets. In the case of the 6-adduct, the ligand is modulated between Arg81 and Arg84 and hence, as the screening of charges intensifies, the local surface is more easily perturbed, leading to two pockets (see Table 2). In the 6'-adduct, the interactions that modulate the conformation of the sugars are those of Trp89 and dipoles of several backbones and polar side chains (and marginally Arg81). Hence, independent of charge screening, the local surface is not much affected, leading to a single large pocket.

Discussion

We know that specificities of enzymes originate in their plastic adaptations to external conditions by combinations

Figure 6. Front view of the binding pockets of TIL with the different ligands (in magenta) docked inside. The protein surface is colored according to the electrostatic potential with blue depicting positively charged and red depicting negatively charged regions. The view is from the *top* looking down into the binding site so the intervening residues have been clipped away to reveal the C α trace of the helical lid region (pink). The substrate in the putative transition states is shown with the lauroyl part in green and the sugar in brown. From *left to right* are structures for $\epsilon = 2$, $\epsilon = 9$, and $\epsilon = 17$. (A) 1-adduct; (B) 6-adduct; (C) 6'-adduct.

of local or/and global structural and dynamic responses (see, e.g., Luo et al. 1998; Miller and Agard 1999; Baladin and Onuchic 2000; Radkiewicz and Brooks 2000; Orencia et al. 2001; Young et al. 2001; Meroueh et al. 2002; Süel et al. 2003). However, the underlying structural and emergent functional complexities have precluded the formulation of any general rules towards an understanding of this plasticity. To further probe the determinants of this plasticity, we have examined, using computational molecular models, how the lipases from two different organisms respond to a ligand under varying environmental conditions. The reaction performed is the transesterification of sucrose, which usually is selective for particular sites on the sugar depending on the enzyme and conditions employed. Molecular modeling is increasingly being recognized as a powerful tool to provide models for the origins of such behavior; its uniqueness over other techniques such as nuclear magnetic resonance, Fourier Transform Infrared spectroscopy, fluo-

rescence resonance energy transfer, lies in simultaneously providing detailed atomic-level information in space and time (Kazlauskas 2001; Warshel 2003; Garcia-Viloca et al. 2004). Although we can only model abstractions of reality, the methods available can provide good qualitative starting points for experimental explorations. Indeed, we (Fuentes et al. 2002; G. Fuentes, A. Ballesteros, and C. Verma, in prep.) and others (Norin et al. 1994; Martinelle et al. 1995; Haeffner et al. 1998; Kazlauskas 2001; Ottosson et al. 2002; Peters and Bywater 2002) have modeled lipase–ligand interactions with encouraging agreement with known experimental observations.

In this study, we have used rigorous conformational sampling (involving energy minimizations of several conformers yielding the enthalpic component of the free energy followed by vibrational entropic computations), because traditional molecular dynamics and energy minimizations only sample limited amounts of the phase space. In contrast, our method allows a much larger sampling of the phase

space by crossing over barriers that would not be sampled otherwise. We examined the extent to which local and global structural plasticity of the lipases modulates their functionalities. Our studies employed crystallographic structures (resolved from aqueous solutions) as the starting points for building the models. This is based on the assumption that the structures of the enzymes in anhydrous organic solvents were essentially identical to the three-dimensional structures of enzymes in water (Fitzpatrick et al. 1993, 1994; Brzozowski et al. 2000). Furthermore, this assumption has worked well in modeling the structure–dynamics–functions of lipases (see, e.g., Kazlauskas 2001; Fuentes et al. 2002; Ottoson et al. 2002).

The overall fold and the catalytic machinery of the two enzymes are very similar, but TIL has a well-defined flexible loop covering the active site, which is linked to its functionality in that it undergoes an activation in the presence of substrates (Brzozowski et al. 2000); no such active lid has been reported for CALB. This is one characteristic feature of many lipases, and although some studies suggest that the time scales of opening and closing this lid seem to be different from the time scales of substrate access (Zandonella et al. 1995), they are not conclusive (Martinelle et al. 1995). What is certain is that the amino acid sequence of the lid affects activity (Brocca et al. 2003). We decided to carry out the modeling studies with the lid in an open position, based on the observation that this methodology has been shown to be successful at reproducing experimental observations (Norin et al. 1994; Fuentes et al. 2002; Ottoson et al. 2002; Peters and Bywater 2002).

The reaction mechanism catalyzed by lipases is believed to be analogous to that of serine proteases. It has been proposed that the tetrahedral intermediate closely resembles the transition state and therefore is a good model for mimicking it (Warshel et al. 1989; Ottoson et al. 2002; Peters and Bywater 2002). During the transesterification reaction, the key intermediates seem to be the deacylation intermediates (Norin et al. 1994), involving both the sucrose and the acyl chains together. Thus, we decided to use this intermediate (to represent the putative transition state) in our work. Of course, our sampling methodology seeks out the lowest energy intermediates, because these mimic the lowest energy putative transition states; the lowest energy transition states will be the experimentally preferred conformations as they will map on to the reaction coordinates with the highest rates. Having identified these intermediates, we investigated the concomitant conformational plasticity and the accompanying structural responses of the enzymes by examining the changes in interatomic distances, the correlations between atomic positional distributions, and the geometric plasticity (areas and volumes) of the binding regions.

From the analyses of the distance matrices it is seen that the binding of the ligands is accompanied by a variety of

structural responses in the enzymes' structures. This serves to illustrate how ligand binding can induce a conformational adaptation, according to the induced-fit theory (Koshland 1958). When the different ligands have diffused within the active site into their minimum energy conformations, the accompanying conformational changes are similar to that seen in other complexes (Done et al. 1998). It appears that residues within the active sites of either enzyme can adopt two distinct and energetically favorable conformations to accommodate the ligands. This is not surprising as it is known that the binding sites in lipases can roughly be partitioned into two regions—a hydrophobic region and a hydrophilic region (Norin et al. 1993). Naturally, the lauroyl moiety “explores” the former, whereas the more hydrophilic sucrose moiety “floats” in the latter.

In the case of CALB, the binding pocket is large and our studies suggest that it is pre-formed to facilitate binding in the unliganded state. The binding region is made up predominantly by hydrophobic (oily) residues. Thus, only minor motions such as small-scale flexing/adaptations of the walls of the binding pocket occur to accommodate the ligands. The binding site is “walled” by a long helix (Glu270–Gly290) whose motions seem important (particularly Ala280–Pro281), irrespective of the dielectric screening. This feature seems to originate in the predominance of hydrophobic residues. Although the local dynamics are quite complex, structural examinations of the low-energy structures suggest that a mutation of Ala281 to Gly would perturb the selectivity of the 6'-adduct (the preferred isomers that are observed are the 6- and 6'-adducts); it is not yet clear whether it would enhance the selectivity or decrease it. A mutation of Ala283 to Ser is likely to enhance the stability of the 1'-adduct by the creation of an additional hydrogen bond between the side chain of Ser and site O₁₁. This is analogous to the observations in other systems such as the dynamics of the proteases seen by Miller and Agard (1999). Mutations of the two Pro residues capping the helix (Pro269, Pro290) to Gly ought to make the region more flexible and alter the distributions of products. In the case of TIL, the binding pocket is less spacious (Pleiss et al. 2000) and hence the fluidity that characterizes the localized dynamics of the binding pocket region in CALB is, in general, not observed. The helical lid that is characteristic of TIL, however, occludes the hydrophobic binding pocket from high dielectric media such as water, and unsurprisingly, undergoes some degree of conformational change to allow the ligands to mould into the catalytic machinery (Brzozowski et al. 2000). This suggests that despite the contrasting time scales of lid dynamics and substrate access (Zandonella et al. 1995), the lid motions control the dynamics of the substrate. In the case of TIL (where selectivity is observed only for the 6-adduct), structural examinations suggest that the selectivity of 1'-adduct might be enthalpically enhanced by mutating Leu259 to Asn, which would stabi-

lize the O₉ and/or O₁₀ sites on the ligand through new hydrogen bonds. Similarly, the 6'-adduct can be stabilized by mutating Gly82 to Ser, which would stabilize O₂ and/or O₃ through new hydrogen bonds. The differing spaces in the binding pockets of the two enzymes would also provide for variations in entropic stabilizations (see, e.g., Ottoson et al. 2002); however, this is beyond the scope of this paper.

The above rearrangements are again seen in analyses of correlations in atomic movements. Such plots are very useful as they reveal how dynamical conformational coupling between regions far apart in structure seemingly affect the binding sites (Sneddon and Brooks 1991; Gerstein et al. 1994; Verma et al. 1997; Radkiewicz and Brooks 2000; Meroueh et al. 2002). CALB is characterized mainly by local couplings, that is, between residues that are close spatially. In stark contrast, we find delineation of the TIL structure into several subdomains/substructures (Figs. 1, 5), which suggests a more global control of ligand binding. The interplay between these flexible subdomains provides structural evidence of the expected dynamic behavior of the surface loops and regions surrounding the catalytic center. Such correlated motions have been postulated, in accordance with experimental observations, to facilitate substrate binding/egress and catalytic modulation (Weston et al. 1992; Rice and Steitz 1994; Newman et al. 1995; Radkiewicz and Brooks 2000; Dvorsky et al. 2002; Gunasekaran and Nussinov 2004). The fact that one side of the binding pocket maps the lid helix, whereas the other sides are made up by regions of the protein that are anti-correlated in motion to the helix, intuitively lends weight to the hypothesis that this kind of anti-correlation corresponds to the expansion/contraction of the binding pocket mouth to facilitate ligand binding/egress (Jääskeläinen et al. 1998b) and catalysis (Dvorsky et al. 2002). Despite the success of such analyses in providing intuitively appealing models, there have been relatively few reports of the effects of mutations on interdomain motions and the associated functionality (van Aalten et al. 1998; Miller and Agard 1999). Examining the structures of TIL, we suggest that the hinge-like region that has Gly109 and Gly177 with C α atoms separated by a distance of 3.7 Å would be a good site for mutations to Ala. Either single or double mutations would enhance the hinge-like property of this region and effect the concerted motions of the regions that encompass the ligand binding site and affect selectivity. Another possible mutation could be Thr143Asn, which would potentially introduce a hydrogen bond between the side chain of Asn and the backbone of Phe13, thus "gluing" the gap between the strand and the helix (containing Phe13). This new hinge region is predicted to affect concerted motions (region 4 in Fig. 5B) and affect reactivity.

The different kinds of functional motions naturally lead to an adapting pocket volume. In CALB, these motions are largely confined to the binding site and only the amino acids

surrounding and creating the pocket display reorientations on the binding of the putative transition state analogs. As expected the lauroyl moiety is stabilized by hydrophobic (and partly neutral) amino acids whereas the sucrose is stabilized by polar interactions; indeed the interactions that are made by the atoms along the lauroyl moiety are quite conservative in that it is known that the chain length can be neither too long (as it would require penetration and destabilization into the protein) nor too short (Ferrer et al. 2000); perhaps some kind of anchoring effect stabilizes the transition state. On the other hand, in TIL, larger secondary structure motions are also involved in the conformational adaptations needed, together leading to delineation of the ligand binding site into multiple pockets. The complexity of the interactions that govern the binding "landscape" can be seen in Figure 6.

Together, the models suggest that optimal binding of the putative transition state requires small-scale local rearrangements of amino acid side chains in a preformed binding site in CALB; in contrast in TIL, the amino acids participating in binding and catalysis seem to undergo substantial reorientation together with secondary structural adjustments. The availability of the larger pocket coupled to a largely local adaptation (and hence defined by a shallow free energy surface) in CALB partly suggests why it catalyzes the formation of two isomers in contrast with TIL, which has a more constrained pocket and requires the mobilization of domains in its functionality (and hence a free energy surface that probably has multiple minima of varying depths) and so leads to the formation of just one isomer. In a low dielectric medium, hydrogen bonding with solvent is expected to be sparse, so the protein organizes itself into 'macro-structures' to compensate for the loss of stability. As the dielectric nature of the surroundings increases, hydrogen bonding with solvent becomes more possible and favorable and these subdomains are mitigated. This, on the one hand, leads to loss of global coupling as mediated by the subdomains but is compensated, on the other hand, by enhanced local plasticity. The two enzymes display a wide spectrum of structural responses to ligand binding at the local or/and the global levels. This manifests itself in the range of selectivities that these enzymes display in their catalytic behaviors. The complexity of the systems makes the formulation of general rules hard; however, some insights can be gained by carrying out computational studies. This leads us to hypothesize that plasticity at the local level modulates functionality through a largely enthalpic control (variations of electrostatic and van der Waals interactions between the substrate and the surrounding amino acids), whereas at a more global level, entropic factors become more important (low-frequency collective motions that are characteristic of domain motions lead to increased entropies and lowering of the free energies) (Fisher et al. 2001; Fuentes et al. 2002). We are investigating these effects in greater detail for these two lipases.

Materials and methods

All of the computations were performed on Silicon Graphics workstations. The manipulations of molecules, the graphic evaluations, the energy minimizations, and the conformational analyses were performed using the molecular modeling program-package QUANTA (Molecular Simulations Inc.) and the program CHARMM (Brooks et al. 1983).

General procedures and starting structures

For our studies, the coordinates for CALB were taken from a 2.6 Å resolution X-ray structure cocrystallized with the inhibitor N-hexylphosphonate ethyl ester (PDB entry 1lbs; Uppenberg et al. 1995), and for TIL, we used the structure with the active-site lid in an open conformation (PDB entry 1dt5; Brzozowski et al. 2000). The CHARMM force field was used to model the protein and water molecules were modeled using the TIP3 potential (Jorgensen et al. 1983). A neutral pH was assumed, leading to net charges of +1 on Arg/Lys side chains and -1 on Asp/Glu side chains, respectively. In addition, the protonated forms of His224 and His258 were used. Optimization of hydrogen bonding potentials (which involved finding the rotamer that made the maximum number of hydrogen bonds) around the side chains of Asn, Gln, and His was done using CHARMM, and the lowest energy structures were selected.

The lauroyl and sucrose were constructed using the molecular editor in QUANTA with partial charges assigned using QUANTA's charge template method. To account for electronic rearrangements in the intermediates, semi-empirical quantum mechanical calculations were performed and the associated changes in the point charges were used to scale the QUANTA-derived charges. This was carried out so that the charges derived for the intermediates were normalized relative to the normal CHARMM-param-

eterized charges for standard amino acids. Coordinates and standard geometries for missing protein atoms were constructed using standard geometries from CHARMM. Nonbonded interactions were truncated at 14 Å with shift and smoothing functions operating between 10 and 14 Å for the electrostatic and van der Waals interactions respectively; constant dielectrics of 2, 9, and 17 were used to represent the different reaction conditions (Ferrer et al. 1999). These values correspond to a conventional organic medium, a medium containing 2-methyl-2-butanol/DMSO 95:5, and a medium with a composition of 2-methyl-2-butanol/DMSO of 80:20, respectively; this has been the approximation chosen for the experimental media used in the synthetic reactions. An initial energy minimization was carried out to remove internal strain using a combination of Steepest Descent (SD) and Adopted Basis Newton Raphson (ABNR) algorithms.

Models for the reaction tetrahedral intermediates

A model for the lipase-lauroyl-sucrose intermediate was constructed by removing the H atom of Ser105 and Ser146 O γ (for CALB and TIL, respectively) and making a covalent link between the Ser105 (or Ser146) O γ , the tetrahedral C (C $_t$) of the lauroyl moiety and the corresponding primary hydroxyl oxygen of the sucrose (O $_6$, O $_1'$, or O $_6'$) (Fig. 7). The resulting tetrahedral carbon C $_t$ and the oxygen atoms were assigned sp 3 hybridization. This protocol was followed for modeling all of the regioisomers in both enzymes.

In the case of CALB, the initial location of the side chain of the ester group was guided by the conformation of the crystallographic inhibitor. The position of the central part of the substrates was well defined because of the requirements of satisfying the hydrogen bonding in the catalytic triad and the formation of the oxyanion hole. For TIL, the protein was structurally aligned with CALB using the residues in the catalytic triad as tethers, and the procedure outlined above was followed.

Figure 7. Structure and nomenclature of the atoms used to model the tetrahedral intermediates and the torsional angles used for the conformational search. (Left) The structure of the lauroyl moiety. The active site serine oxygen of the lipase is the oxygen (not bolded) linked to the atom C1 shown in the lauroyl. The oxygen atom in boldface type and with a * is the oxygen at which the esterification reaction involving addition of the sucrose isomers (middle) takes place; the arrows in the middle point to the 3 different sites of esterification (leading to the three different products that are discussed). (Right) The various torsional angles that were varied in the sucrose moiety (as shown in the middle of the figure) during the conformational searches.

Systematic conformational search

There are two methods that can be used to explore the conformational plasticity of large molecules such as proteins: static, grid-based conformational searches for isolating the minima and molecular dynamics simulations. To sample the possible conformations of protein–substrate complexes, the use of molecular dynamics is inappropriate because the time scales required to sample the various conformations will be too long to be simulated on current computers. On the other hand, it is not possible to search the total conformational space of an enzyme–substrate system of the size of lipases using the static grid-based methods either. Thus, we chose to perform a local grid-based conformational search. For this, the torsion angles around the five bonds shown in Figure 7 were varied systematically in each complex and the resulting conformations were energy minimized. These angles were varied from -60° to 180° with a grid size of 120° .

The resulting 243 conformations were minimized following a protocol based on decremental constraints that apply to the protein as follows: Two segments were defined, segment **a**, formed by the residue 106 (or 146) belonging to the protein chain A, and segment **b** with the *n*-laurate and sucrose moieties. Initially, a harmonic force of 20.0 kcal/mol/Å was applied to the whole system excluding the two segments, the hydrogen atoms, and water molecules. Segment **a** was constrained with a force constant of 5.0 kcal/mol/Å, and segment **b** with 1.0 kcal/mol/Å. The system was subjected to 200 steps of Steepest Descent and 800 steps of Adopted Basic Newton Raphson minimization. Subsequent cycles of minimization were carried out, gradually decreasing the harmonic force imposed on the system; finally, the unconstrained system was subject to 200 steps of SD and 5000 steps of ABNR minimization, until the energy gradient was less or equal to 0.01 kcal/mol/Å. This method has the advantage of sampling several conformations of the substrate and hence enables identification of complexes of interest much more quickly than is possible from molecular dynamics simulations.

Analysis

The minimum energy structures for each combination of dielectric and acylation site were taken together for further analysis. This was carried out by computing difference distance matrices, correlation plots, and pocket plasticities. For the difference distance matrices, the inter-C α distances in each structure were computed and the change in this index between different minimum energy structures was examined. For the correlated plots (Ichiye and Karplus 1991), the correlation between the distributions of C α positions in all minimum energy structures for each enzyme was separately computed. The plasticity of the enzyme pockets was computed through their areas and volumes, using the server CASTp (Liang et al. 1998). This program uses weighted Delaunay triangulations and is also based on the pocket algorithm of alpha shape theory. It provides identification and measurements of surface-accessible pockets as well as interior-inaccessible cavities (defining a cavity as the interior empty space that is not accessible to the solvent probe of radius 1.4 Å), and it measures analytically the area and volume of each pocket and cavity.

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References

- Bacon, D.J. and Moulton, J. 1992. Docking by least-squares fitting of molecular surface patterns. *J. Mol. Biol.* **225**: 849–858.
- Baladin, I.A. and Onuchic, J. 2000. Dynamically controlled protein tunneling paths in photosynthetic reaction centers. *Science* **290**: 114–117.
- Brocca, S., Secundo, F., Ossola, M., Alberghina, L., Carrea, G., and Lotti, M. 2003. Sequence of the lid affects activity and specificity of *Candida rugosa* lipase isoenzymes. *Protein Sci.* **12**: 2312–2319.
- Brooks, B.R., Brucoleri, R.E., Olafson, B.D., States, D.J., Swaminathan, S., and Karplus, M. 1983. CHARMM: A program for macromolecular energy minimization and dynamics calculations. *J. Comput. Chem.* **4**: 187–217.
- Brzozowski, A.M., Savage, H., Verma, C.S., Turkenburg, J.P., Lawson, D.M., Svendsen, A., and Patkar, S. 2000. Structural origins of the interfacial activation in *Thermomyces* (Humicola) lanuginosa lipase. *Biochemistry* **39**: 15071–15082.
- Done, S.H., Brannigan, J.A., Moody, P.C.E., and Hubbard, R.E. 1998. Ligand-induced conformational change in penicillin acylase. *J. Mol. Biol.* **284**: 463–475.
- Dvorsky, R., Hornak, V., Sevcik, J., Tyrrell, G.P., Caves, L.S.D., and Verma, C.S. 2002. Dynamics of Rnase-Sa: A simulation perspective complementary to NMR/X-ray. *J. Phys. Chem. B* **106**: 6038–6048.
- Elcock, A.H. 2003. Atomic-level observation of macromolecular crowding effects: Escape of a protein from the GroEL cage. *Proc. Natl. Acad. Sci.* **100**: 2340–2344.
- Faber, H.R. and Matthews, B.W. 1990. A mutant T4 lysozyme displays five different crystal conformations. *Nature* **348**: 263–266.
- Ferrer, M., Cruces, M.A., Bernabé, M., Ballesteros, A., and Plou, F.J. 1999. Lipase-catalyzed regioselective acylation of sucrose in two-solvent mixtures. *Biotechnol. Bioeng* **65**: 10–15.
- Ferrer, M., Cruces, M.A., Plou, F.J., Bernabé, M., and Ballesteros, A. 2000. A simple procedure for the regioselective synthesis of fatty acid esters of maltose, leucrose, maltotriose and *n*-dodecyl maltosides. *Tetrahedron* **56**: 4053–4061.
- Fischer, S., Smith, J.C., and Verma, C.S. 2001. Dissecting the vibrational entropy change on protein/ligand binding: Burial of a water molecule in bovine pancreatic trypsin inhibitor. *J. Phys. Chem. B* **105**: 8050–8055.
- Fitzpatrick, P.A., Steinmetz, A.C.U., Ringe, D., and Klivanov, A.M. 1993. Enzyme crystal-structure in a neat organic solvent. *Proc. Natl. Acad. Sci.* **90**: 8653–8657.
- Fitzpatrick, P.A., Ringe, D., and Klivanov, A.M. 1994. X-ray crystal structure of cross-linked subtilisin Carlsberg in water vs. acetonitrile. *Biochem. Biophys. Res. Comm.* **198**: 675–681.
- Frauenfelder, H. and McMahon, B. 1998. Dynamics and function of proteins: The search for general concepts. *Proc. Natl. Acad. Sci.* **95**: 4795–4797.
- Frauenfelder, H., McMahon, B.H., Austin, R.H., Chu, K., and Groves, J.T. 2001. The role of structure, energy landscape, dynamics, and allostery in the enzymatic function of myoglobin. *Proc. Natl. Acad. Sci.* **98**: 2370–2374.
- Fuentes, G., Cruces, M.A., Plou, F.J., Ballesteros, A., and Verma, C.S. 2002. Computational studies of subtilisin-catalyzed transesterification of sucrose: Importance of entropic effects. *ChemBioChem* **3**: 907–910.
- Garcia-Viloca, M., Gao, J., Karplus, M., and Truhlar, D.G. 2004. How enzymes work: Analysis by modern rate theory and computer simulations. *Science* **303**: 186–195.
- Gerstein, M., Lesk, A., and Chotia, C. 1994. Structural mechanisms for domain movements in proteins. *Biochemistry* **33**: 6739–6749.
- Goodsell, D.S. and Olsen, A.J. 1990. Automated docking to proteins by simulated annealing. *Proteins* **8**: 195–202.
- Gunasekaran, K. and Nussinov, R. 2004. Modulating functional loop movements: The role of highly conserved residues in the correlated loop motions. *ChemBioChem* **5**: 224–230.
- Haeflner, F., Norin, T., and Hult, K. 1998. Molecular modeling of the enantioselectivity in lipase-catalyzed transesterification reactions. *Biophys. J.* **74**: 1251–1262.
- Ichiye, T. and Karplus, M. 1991. Collective motions in proteins: A covariance analysis of atomic fluctuations in molecular dynamics and normal mode simulations. *Proteins* **11**: 205–217.
- Jääskeläinen, S., Verma, C.S., Hubbard, R.E., and Caves, L.S.D. 1998a. Identifying key electrostatic interactions in *Rhizomucor miehei* lipase: The influence of solvent dielectric. *Theor. Chem. Acc.* **101**: 175–179.
- Jääskeläinen, S., Verma, C.S., Hubbard, R.E., Linko, P., and Caves, L.S.D. 1998b. Probing the conformational change in the activation of lipase. *Protein Sci.* **7**: 1359–1367.
- Johnson, L.N., Stura, E.A., Wilson, K.S., Sansom, M.S.P., and Weber, L.T.

1979. Nucleotide binding to glycogen phosphorylase b in the crystal. *J. Mol. Biol.* **134**: 639–653.
- Jones, S. and Thornton, J.M. 1996. Principles of protein–protein interactions derived from structural studies. *Proc. Natl. Acad. Sci.* **93**: 13–20.
- Jorgensen, W.L., Chandrasekhar, J., Madura, J.D., Impey, R.W., and Klein, M.L. 1983. Comparison of simple potential functions for simulating liquid water. *J. Chem. Phys.* **79**: 926–935.
- Kazlauskas, R.J. 2001. Modeling—A tool for experimentalists. *Science* **293**: 2277–2279.
- Koshland, D.E. 1958. Application of a theory of enzyme specificity to protein synthesis. *Proc. Natl. Acad. Sci.* **44**: 98–104.
- Liang, J., Edelsbrunner, H., and Woodward, C. 1998. Anatomy of protein pockets and cavities: Measurements of binding site geometry and implications for ligand design. *Protein Sci.* **7**: 1884–1897.
- Luo, X., Kato, R., and Collins, J.R. 1998. Dynamic flexibility of protein-inhibitor complexes: A study of the HIV-1 protease/KNI-272 complex. *J. Amer. Chem. Soc.* **120**: 12410–12418.
- Martinelle, M., Holmquist, M., and Hult, K. 1995. On the interfacial activation of *Candida antarctica* lipase A and B as compared with *Humicola lanuginosa* lipase. *Biochim. Biophys. Acta* **1258**: 272–276.
- McCammon, J.A. and Harvey, S.C. 1987. *Dynamics of proteins and nucleic acids*. Cambridge University Press, Cambridge, UK.
- Meroueh, S.O., Roblin, P., Golemi, D., Maveyraud, L., Vakulenko, S.B., Zhang, Y., Samama, J.-P., and Mobashery, S. 2002. Molecular dynamics at the root of expansion of function in the M69L inhibitor-resistant TEM -lactamase from *Escherichia coli*. *J. Am. Chem. Soc.* **124**: 9422–9430.
- Miller, D.W. and Agard, D.A. 1999. Enzyme specificity under dynamic control: A normal mode analysis of α -lytic protease. *J. Mol. Biol.* **286**: 267–278.
- Mulder, F.A., Mittermaier, A., Hon, B., Dahlquist, F.W., and Kay, L.E. 2001. Studying excited states of proteins by NMR spectroscopy. *Nat. Struct. Biol.* **8**: 932–935.
- Newman, M., Strzelecka, T., Dorner, L.F., Schildkraut, I., and Aggarwal, A.K. 1995. Structure of bam-hi endonuclease bound to DNA—partial folding and unfolding on DNA-binding. *Science* **269**: 656–663.
- Norin, M., Haeflner, F., Achour, A., Norin, T., and Hult, K. 1994. Computer modeling of substrate-binding to lipases from *Rhizomucor miehei*, *Humicola lanuginosa* and *Candida rugosa*. *Protein Sci.* **3**: 1493–1503.
- Orencia, M.C., Yoon, J.S., Ness, J.E., Stemmer, W.P.C., and Stevens, R.C. 2001. Predicting the emergence of antibiotic resistance by directed evolution and structural analysis. *Nat. Struct. Biol.* **8**: 238–242.
- Otto, R.T., Scheib, H., Bornscheuer, U.T., Pleiss, J., Sydlatk, C., and Schmid, R.D. 2000. Substrate specificity of lipase B from *Candida antarctica* in the synthesis of arylaliphatic glycolipids. *J. Mol. Catal. B: Enzym.* **8**: 201–211.
- Ottosson, J., Fransson, L., and Hult, K. 2002. Substrate entropy in enzyme enantioselectivity: An experimental and molecular modeling study of a lipase. *Protein Sci.* **11**: 1462–1471.
- Pandey, A., Benjamin, S., Soccol, C.R., Nigam, P., Krieger, N., and Soccol, V.T. 1999. The realm of microbial lipases in biotechnology. *Biotechnol. Appl. Biochem.* **29**: 119–131.
- Pauling, L. 1948. Nature of the forces between large molecules of biological interest. *Nature* **161**: 707–709.
- Peters, G.H. and Bywater, R.P. 2002. Essential motions in a fungal lipase with bound substrate, covalently attached inhibitor and product. *J. Mol. Recognit.* **15**: 393–404.
- Pleiss, J., Fischer, M., and Schmid, R.D. 1998. Anatomy of lipase binding sites: The scissile fatty acid binding site. *Chem. Phys. Lipids* **93**: 67–80.
- Pleiss, J., Fischer, M., Peiker, M., Thiele, C., and Schmid, R.D. 2000. Lipase engineering database: Understanding and exploiting sequence–structure–function relationships. *J. Mol. Catal. B: Enzym.* **10**: 491–508.
- Radkiewicz, J.L. and Brooks, C.L. 2000. Protein dynamics in enzymatic catalysis: Exploration of dihydrofolate reductase. *J. Amer. Chem. Soc.* **122**: 225–231.
- Rice, P.A. and Steitz, T.A. 1994. Refinement of γ - δ -resolvase reveals a strikingly flexible molecule. *Structure* **2**: 371–384.
- Schmid, R.D. and Verger, R. 1998. Lipases: Interfacial enzymes with attractive applications. *Angew. Chem. Int. Ed. Eng.* **37**: 1609–1633.
- Sneddon, S.F. and Brooks, C.L. 1991. Protein motions: Structural and functional aspects. In *Molecular structures in biology* (eds. R. Diamond et al.), pp. 113–160. Oxford University Press, Oxford, UK.
- Süel, G.M., Lockless, S.W., Wall, M.A., and Ranganathan, R. 2003. Evolutionarily conserved networks of residues mediate allosteric communication in proteins. *Nat. Struct. Biol.* **10**: 59–69.
- Uppenberg, J., Ohnner, N., Norin, M., Hult, K., Kleywegt, G.J., Patkar, S., Waagen, V., Anthonsen, T., and Jones, T.A. 1995. Crystallographic and molecular-modeling studies of lipase B from *Candida antarctica* reveal a stereospecificity pocket for secondary alcohols. *Biochemistry* **34**: 16838–16851.
- van Aalten, D.M.F., Hoff, W.D., Findlay, J.B.C., Crielard, W., and Hellingwerf, K.J. 1998. Concerted motions in the photoactive yellow protein. *Prot. Eng.* **11**: 873–879.
- Verma, C.S., Caves, L.S.D., Hubbard, R.E., and Roberts, G.C.K. 1997. Domain motions in dihydrofolate reductase: A molecular dynamics study. *J. Mol. Biol.* **226**: 776–796.
- Warshel, A. 2003. Computer simulations of enzyme catalysis: Methods, progress, and insights. *Annu. Rev. Biophys. Biomol. Struct.* **32**: 425–443.
- Warshel, A., Naray-Szabo, G., Sussman, F., and Hwang, J.K. 1989. How do serine proteases really work? *Biochemistry* **28**: 3629–3637.
- Weston, S.A., Lahm, A., and Suck, D. 1992. X-ray structure of the DNase I-D(GGTATACC)(2) complex at 2.3-Å resolution. *J. Mol. Biol.* **226**: 1237–1256.
- Woudenberg, M., Van Rantwijk, F., and Sheldon, R.A. 1996. Regioselective acylation of disaccharides in tert-butyl alcohol catalyzed by *C. antarctica* lipase. *Biotechnol. Bioeng.* **49**: 328–333.
- Young, M.A., Gonfloni, S., Superti-Furga, G., Roux, B., and Kuriyan, J. 2001. Dynamic coupling between the SH2 and SH3 domains of c-Src and Hck underlies their inactivation by C-terminal tyrosine phosphorylation. *Cell* **105**: 115–126.
- Zandonella, G., Haalck, L., Spener, F., Faber, K., Paltauf, F., and Hermetter, A. 1995. Inversion of lipase stereospecificity for fluorogenic alkylacyl glycerols. Effect of substrate solubilization. *Eur. J. Biochem.* **231**: 50–55.