Computational Modeling of a Biocatalyst at a Hydrophobic Substrate Interface

Sven Benson and Jürgen Pleiss

Abstract Solvent molecules play a crucial role in the function of proteins. The solvent flux method (SFM) was developed to comprehensively characterize the influx of solvent molecules from the solvent environment into the active site of a protein by molecular dynamics simulations. This was achieved by introducing a solvent concentration gradient and by reorienting and rescaling the velocity vector of all solvent molecules contained within a spherical volume enclosing the protein, thus inducing an accelerated solvent influx toward the active site. In addition to the detection of solvent access pathway within the protein structure, it is hereby possible to identify potential amino acid positions relevant to solvent-related enzyme engineering with high statistical significance. The method is particularly aimed at improving the reverse hydrolysis reaction rates in nonaqueous media. Candida antarctica lipase B (CALB) binds to a triglyceride-water interface with its substrate entrance channel oriented toward the hydrophobic substrate interface. The lipase-triglyceride-water system served as a model system for SFM to evaluate the influx of water molecules to the active site. As a proof of principle for SFM, a previously known water access pathway in CALB was identified as the primary water channel. In addition, a secondary water channel and two pathways for water access which contribute to water leakage between the protein and the triglyceride-water interface were identified.

1 Introduction

The first pioneering studies of an esterase in a non-aqueous solvent were performed 75 years ago, but it was not until the 1980s that the synthetic opportunities of enzymes in organic solvents were realized. Multiple studies confirmed that the catalytic activity sensitively depends on the water content. Water content and solvent polarity were identified as the major determinants of catalytic activity and stereoselectivity. It was suggested that the effect of organic solvents on an

S. Benson • J. Pleiss (✉)
Institute of Technical Biochemistry, University of Stuttgart, Allmandring 31, 70569 Stuttgart, Germany
e-mail: Juergen.Pleiss@itb.uni-stuttgart.de

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enzyme is primarily caused by the interactions between the solvent and the enzyme-bound water rather than with the enzyme itself, and that a low amount of water is required to provide sufficient conformational flexibility to the enzyme. To explore the molecular basis of enzyme stability, solubility, catalytic activity, substrate specificity, and selectivity, the effect of solvents to protein structure and dynamics was investigated by computational modelling. A deep understanding of protein packing, the structure of the active site, and the contribution of protein motions to function is prerequisite to a successful design of enzymes, the prediction of mutants, and the choice of appropriate solvents.

2 Enzymes at Substrate Interfaces

Water molecules are directly involved in many enzymatic reactions such as hydrolysis reactions catalyzed by esterase and lipases. While this natural degradative enzyme function is of substantial interest to a range of industrial applications, significant strides have been made during the last decades to shift the chemical equilibrium towards the reverse synthesis reaction by transferring hydrolases into non-natural organic solvent environments. The presence of residual hydration water is a prerequisite for enzyme activity in non-aqueous environments. Moreover, defining and maintaining dry conditions is difficult as well as costly to achieve on the industrial scale, which motivated Larsen [13] to propose an approach to suppress water as a nucleophile in CALB by rationally designing variants that diminish the influx of water to the active site cavity and thus dramatically increase transacylation rates for vinyl butyrate over hydrolysis rates in butanol-water mixtures. CALB catalyzes hydrolysis of triglycerides in its native physiological setting; the natural substrate triglyceride is water-insoluble and forms phase-separated interfaces in aqueous environments and thus CALB attaches to these interfaces in its active conformation, which is hereby oriented with its substrate channel towards the triglyceride phase [6]. The most obvious water access pathway is thus considered to be blocked by the triglyceride which forms a hydrophobic barrier. However, a constant influx of water to the active site cavity is a prerequisite for hydrolysis to occur. Therefore, Larsen [13] proposed the existence of a water channel, which was targeted for mutation and implicitly validated by experiment. Due to the difficulty of examining individual water molecules by experimental means, molecular dynamics (MD) studies have been conducted extensively to elucidate enzyme-solvent interactions under various conditions, which are comprehensively summarized in a recent review [14]. By analyzing hydration water and its influence on protein secondary structure flexibility, it may also be possible to indirectly identify water entrance channels [4]. In the present study, we propose an MD-based approach, the solvent flux method (SFM), which allows for a holistic characterization of the influx of solvent molecules through the protein structure and into the active site cavity. In this context, SFM was benchmarked for the influx of water molecules to the active

site of CALB by comparing in silico predictions to the experimental results by Larsen [13].

3 Simulation Details

More than $1\mu s$ of MD simulations were conducted on a system consisting of 100,000 atoms using the Hermit cluster, a Cray XE6 system of the High Performance Computing Center (HLRS) in Stuttgart, Germany. Over 300 simulations, including benchmark and test runs, were performed on 256 cores in parallel. The GROMACS 4.5.3 [9, 22] software was used to model an NPT ensemble at 298.15 K and $1bar$, applying the leap-frog integrator [23] with a time step of $2fs$. The Berendsen thermo- and barostats were applied for their robustness and efficiency during equilibration. This was necessary since the SFM method introduces significant periodic rescaling and reorientation of water molecule velocity vectors close to the protein and thus drives the systems out of equilibrium. Water molecules with adjusted velocity vectors were loosely coupled with a relaxation time of 50 ps to prolong the velocity rescaling effect, while the remaining bulk water molecules, the triglyceride molecules, and the protein were coupled tightly in intervals of 0.1 ps. To ensure that the structural integrity of the system remained intact during simulation and to avoid protein denaturation or distortions in the protein-triglyceride interface, position restraints with force constants of $1000\,\mathrm{kJmol^{-1}nm^{-2}}$ were applied to the protein backbone and the triglyceride molecules in all spatial dimensions. To avoid artifacts due to position restraints under NPT conditions, the reference coordinates of each molecule group's center of mass were rescaled periodically. Pressure coupling was applied separately to the x-y plane and in z-dimension for a more accurate pressure representation of the planar triglyceride layer [17] in the model system. Pressure coupling intervals were set to 100 ps for the adjusted water molecules and to 5 ps for the protein, triglyceride and bulk water molecules. The center of mass movements were removed every 100 simulation steps independently for all system components. Periodic boundary conditions were applied in all three dimensions. Hydrogen bonds were constrained with the LINCS algorithm [18]. Long-range electrostatics were calculated with the particle mesh Ewald method [5]. Lennard-Jones interactions were treated with a cutoff and capped at 1.2 nm. The OPLS-AA all-atom force field [11] was used to parameterize CALB. Structural information of CALB was obtained from the Protein Data Bank (PDB: 1TCA [21]). Triglyceride molecules were parameterized with the Berger lipid model [3] and the 1–4 interactions adapted with the half-ϵ double-pairlist method (Neale and Pomès, 2011, Combination rules for united-atom lipids and oplsaa proteins. http://www.pomeslab.com/files/lipidCombinationRules.pdf. Accessed 2 March 2011, Unpublished document) for consistency with the OPLS-AA force field. Water molecules were parameterized with the TIP3P [10] model.

4 Modeling of Candida antarctica Lipase B Attached to a Triglyceride Layer

A model system was created to mimic the conditions of the functionally active conformation of CALB at the triglyceride-water interface. As a prerequisite step, a random molecular arrangement of caproic (C6) triglyceride molecules and water molecules had to be equilibrated into a phase-separated triglyceride layer, in accordance to the approach proposed by Gruber [6]. The resulting triglyceride-water interface hereby represents a model for the surface of large-scale aggregates in triglyceride in water emulsions (Fig. 1). This model was deemed appropriate considering that the diameter of CALB ($\approx$5 nm) is three orders of magnitude smaller than experimentally determined triglyceride droplet diameters ($\approx$2.0 μm) [12]; hence the droplet surface curvature is expected to be negligible on the model scale. Furthermore, when considering that the Coulomb potential decays with $1/r$, a layer thickness of $\approx$7 nm should be sizeable enough to ensure that the most significant Coulomb contributions to the non-bonded potential between the protein and the triglyceride surface are accounted for. Moreover, the lamellar-like nanostructures observed for the triglyceride layer model are in agreement with previously published results on large-scale triglyceride aggregates [19] and our findings reported in a previous study [1]. The potential implication of these structural features on the mobility of water molecules are subject to a detailed follow-up study and will therefore not be elaborated on in this context. CALB was added to the triglyceride-water system and was attached to the layer with the active site entrance pointing towards the triglyceride phase, thus representing a model for the active binding

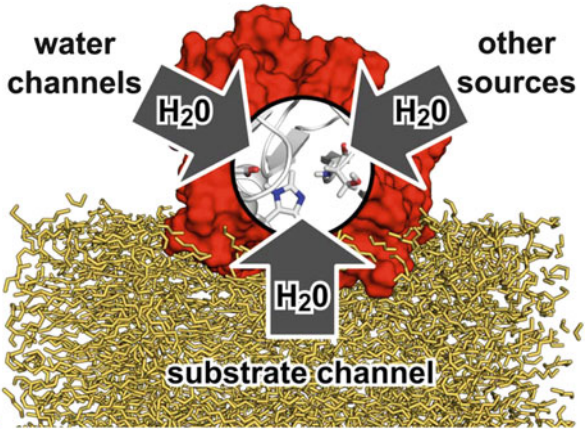

Fig. 1 The protein structure of Candida antarctica Lipase B (PDB: 1TCA) was adsorbed at a surface model of its natural substrate triglyceride (caproic (C6) fatty acid chain length). During equilibration, the protein immersed into the substrate layer, its main entrance being covered by the hydrophobic triglyceride molecule phase. This system served as the basis to model the water influx from potential sources. Reprinted with permission, copyright 2014 American Chemical Society[2]

configuration for triglyceride hydrolysis. The system was equilibrated for a further 500 ns, while monitoring protein adhesion and immersion depth (unpublished data).

5 Modeling the Water Influx into the Active Site of Candida antarctica Lipase B at a Triglyceride-Water Interface

The design and benchmarking of SFM was tightly coupled to the test case system of protein CALB attached to the triglyceride-water interface and using water as the influx solvent. Two major technical considerations were formative for SFM during the design phase. Firstly, it was considered necessary to differentiate solvent molecules that pass through a water access pathway into the active site cavity from other bulk solvent molecules. This was achieved in a recursive manner, by exploiting the fact that solvent molecules that are present within the active site cavity necessarily have to have passed through a water access pathway of the protein at a preceding point in time. It was thus possible to reconstruct solvent molecule pathways through the protein and therefore to detect general access pathways on a statistical basis. To identify such water molecules and to ensure that they had indeed passed into the active site cavity from the exterior, the water molecule closest to the reference atom at the intended influx site (here: oxygen atom of Ser105 of the catalytic triad of CALB) and within a spherical water removal cutoff of 0.35 nm was periodically removed from the simulation system after $\Delta t_{ITER} = 10$ ps (Fig. 2). Not only did this ensure that after a given number of iterations all solvent molecules previously present within the active site cavity were evacuated, it also introduced an increased influx of solvent molecules due to the solvent concentration gradient between the active site of the protein and the exterior solvent bulk. Moreover, the removal of water served as a mechanistic model for water consumption during the native CALB hydrolysis reaction. The induced solvent influx ties in with the second major technical consideration, which is the acceleration of conventional MD to rapidly overcome rate-limiting energy barriers specific to water influx and thus realize a significant data depth in a feasible duration of real-time. This was achieved by introducing a reorientation and rescaling of velocity vectors of water molecules that were located within a water velocity rescaling cutoff of 3 nm surrounding the active site reference, which was adjusted to incorporate the entirety of the protein and its first solvation shell. Within this cutoff, the velocity vectors of water molecules were periodically readjusted at constant time intervals Δt_{ITER}, simultaneously to the removal of water molecules. Velocity magnitudes of water molecules present within this cutoff were rescaled with a constant factor v_{MULT}, which effectively defines the strength of a periodic velocity pulse that is introduced to overcome the influx-rate-limiting energy barriers in a timely manner. The orientation of the new vectors was defined by assigning a fraction v_{OLD} of the respectively rescaled vector magnitudes to the unit vectors in the original direction, while a larger fraction v_{CENT}=100 %−v_{OLD} was assigned to unit vectors pointing towards the active site reference (Fig. 2). Summation of these vector pairs yielded

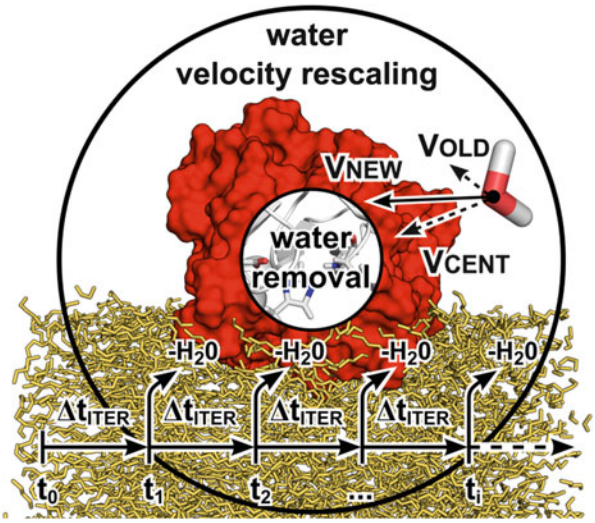

Fig. 2 Velocity vector magnitudes of water molecules found within the water velocity rescaling cutoff around an active site reference atom are rescaled by a factor of vMULT and then transformed to v_{NEW} after every iteration Δt_{ITER}. After every iteration Δt_{ITER}, a single water molecule is removed within the water removal cutoff radius. The direction of v_{NEW} is defined by X% of the rescaled magnitudes that are allocated to the original vector directions (v_{OLD}) and by Y% of the rescaled magnitudes that are allocated to the direction of the active site reference atom (v_{CENT}), whereby $X+Y = 100$ and $Y \gg X$. Reprinted with permission, copyright 2014 American Chemical Society[2]

the new velocity vectors v_{NEW}, which were used to update the initial conditions at the beginning of an iteration. Thereafter, temperatures were allowed to converge back towards equilibrium during Δt_{ITER}. It proved necessary to additionally fine-tune the duration of this effect by modulating the temperature coupling parameter τ_{ITER}, thus coupling the velocity-adjusted water molecules more loosely than other system components. The process of single water molecule removal, velocity readjustment and simulation continuation was repeated for a predefined number of iteration steps n_{ITER}. With ΔN_{WAT} denoting the sum of all water molecules removed during a full SFM run, it was thus possible to express the total water removal rate at the active site reference as $\Delta N_{WAT} / n_{ITER}$, $\Delta N_{WAT} / n_{ITER}$=1 signifying water removal at every iteration.

6 Applying the Solvent Flux Method to Candida antarctica Lipase B

For the case study of the enzyme Candida antarctica lipase B (CALB) with the solvent water the aforementioned SFM parameters were adjusted in a thorough benchmarking to both optimize the solvent flux $\Delta N_{WAT} / n_{ITER} = 1$ as well as

maintain a stable simulation system. The feasibility of SFM analysis was then assessed by comparing simulation results with the successful experimental study of Larsen on the same system [13], wherein enzyme variants were engineered that interfere with water access and thus increase transacylation versus hydrolysis rates. Water influx towards the active site reference oxygen atom of Ser105 of CALB was induced by SFM, due to the water concentration gradient between the protein exterior and the active site, induced by the periodic removal of water molecules and the periodic introduction of a radial velocity pulse to the water molecules in close proximity to the protein. After an iteration $n_{EVAC} = 100$, it was ensured that all water molecules that were originally present in the active site cavity had been removed and thus any water molecules that were consecutively removed in $n_i > n_{EVAC}$ necessarily had to have passed through a water access pathway of the protein to reach the active site. The pathway of any water molecule that was thus removed during an iteration *ni*, with $n_{EVAC} < n_i < n_{ITER} = 500$, was reconstructed by superimposing the system coordinates in a least square fit of protein backbone atoms of all prior iterations $n_j < n_i$. Moreover, the contact frequency between the removed water molecules and all amino acids was evaluated on the basis of the reconstructed water pathways by counting the number of iterations during which a water molecule was found within the contact distance $r_{HSPOT} = 0.4$ nm to a specific amino acid. The SFM analysis was applied to three different initial conformations of the model system (Fig. 1) which were derived from a prior 100*ns* MD simulation under conventional equilibrium conditions without external forces or water removal, whereby the starting coordinates corresponded to the minimal, the maximal and an average RMSD value of the CALB protein backbone. For each initial conformation, SFM was performed 50 times for a total of 150 independent runs, each consisting of 500 iterations of $\Delta t_{ITER} = 10$ ps, amounting to a total of 750 ns of accelerated SFM analysis. Contact frequencies were extracted from this data set, normalized and merged for a holistic and statistically significant representation of probable water access pathways during influx to the active site cavity of CALB.

7 Water Access Channels and Critical Amino Acid Positions Identified in Candida antarctica Lipase B by the Solvent Flux Method

Amino acid positions corresponding to increased contact frequencies were defined as hotspot areas H1-H9 (Fig. 3). Individual hotspot positions (Fig. 4) contained in hotspot areas were defined on the basis of their contact frequency, their relative position within the CALB structure and by visualizing reconstructed water influx pathways (Fig. 5). Hotspot positions were thereby predominantly found in close proximity to the triglyceride-water interface. Positions Thr42/Ser47 of H1 and Gln106 of H3 coincide with the mutational strategy on CALB of Larsen [13], who proposed and implicitly validated a water channel at these exact positions. Because

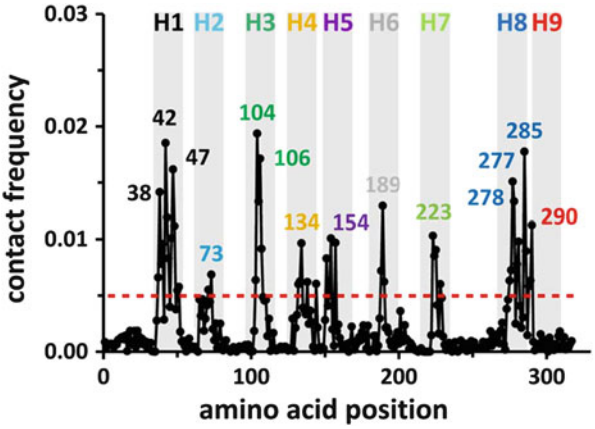

Fig. 3 Contact frequency between influx water and position-specific amino acid are presented as the number of observed water contacts relative to all evaluated system conformations of the SFM analysis (all potential contacts). Hotspot areas H1-H9 were defined as peaks greater than a contact frequency cutoff value (*red dotted line*) and were arbitrarily colored for reasons of easy distinction and reference in Figs. 4 and 5. Single positions that correspond to the highest values of the hotspot areas were labeled and defined as hotspot positions. Reprinted with permission, copyright 2014 American Chemical Society[2]

	pos.	AA	property	rel. freq.	d_{act} [Å]	positioning	assessment	chan
	38	PRO	hphob	0.71	5.7	periphery	adjacent to reported water channel	-
H1	42	THR	hphil	1.00	7.8	periphery	reported water channel	Larson
	47	SER	hphil	0.85	8.5	periphery	reported water channel	Larson
H2	73	LEU	hphob	0.27	10.8	surface	probable barrier, adjacent to Lys290	-
H3	104	TRP	hphob	0.96	4.8	active site	active site	-
	106	GLN	hphil	0.98	4.8	active site	reported water channel	Larson
H4	134	ASP	neg	0.54	11.5	periphery	potential secondary water entrance	S1
H5	154	VAL	hphob	0.56	12.0	periphery	entrance substrate channel	-
H6	189	ILE	hphob	0.96	9.4	surface	entrance substrate channel	-
H7	223	ASP	neg	0.65	12.7	surface	potential secondary water entrance	S2
	277	LEU	hphob	0.77	8.7	periphery	probable barrier, adj. to Asp223 and Ser47	
H8	278	LEU	hphob	0.88	6.9	periphery	entrance substrate channel	
	285	ILE	hphob	0.94	11.3	periphery	entrance substrate channel	
H9	290	LYS	pos	0.52	17.7	surface	potential secondary water entrance	S3

Fig. 4 Compiled hotspot areas H1-H9 obtained from 150 SFM runs analyzing water influx into CALB attached to a triglyceride-water interface depicted in Fig. 1. Data shown includes amino acid positions (pos.), amino acids (AA), amino acid properties in regards to charge and hydrophobicity and hydrophilicity (property), the relative contact frequency calculated by SFM (rel. freq.), the distance (d_{act}) of residue Cα carbon atoms relative to the oxygen reference atom of Ser105 at the active site, the spatial positioning of the amino acid in the protein structure (positioning), a general assessment of the individual hotspot positions (assessment), and a channel definition (channel), whereby the primary water channel (PWC) is the water channel reported by Larsen [13], the secondary water channel (SWC) a potential secondary water channel, and two interfacial water entrances (IWE1, IWE2) located at the protein-triglyceride interface that may contribute to the water influx via the substrate channel

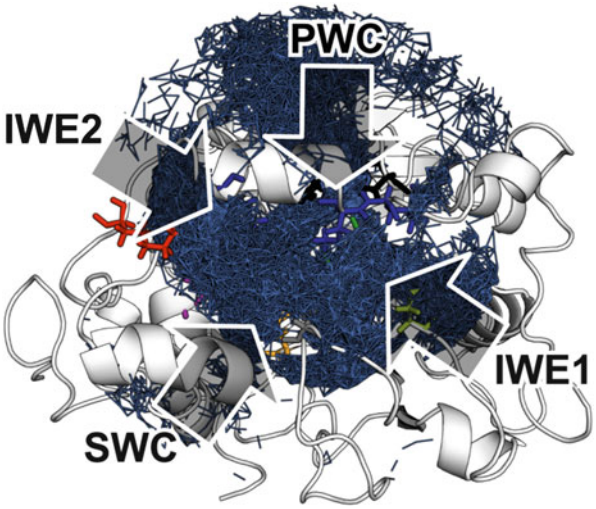

Fig. 5 Water influx pathways from five full SFM runs of 500 iterations length are superimposed onto the same depiction to illustrate potential water access pathways (*arrows*). Helices between the PWC and SWC channels and the triglyceride layer stop water "leakage" in between the protein and the interface, whereas IWE1 and IWE2 significantly contribute to the influx via the substrate channel, which is situated in the central part of the protein in both depictions. Reprinted with permission, copyright 2014 American Chemical Society[2]

it had the highest relative contact frequency (Fig. 4), we refer to this channel as the primary water channel (PWC). Three additional potential water access pathways with high relative contact frequencies were identified, the secondary water channel (SWC) at position Val134 of H4 and two interfacial water entrances IWE1 and IWE2 at position Asp223 of H7 and Lys290 of H9, respectively, which feature polar residues on the protein surface and are in immediate contact with the triglyceride interface. IWE1 and IWE2 are therefore considered to potentially contribute to the significant water influx through the substrate channel leading from the triglyceride interface to the active site (Fig. 5), which is lined by positions Val154 of H5, Ile189 of H6, and Leu278/Ile285 of H8. Other potential hotspot positions, such as Pro38 of H1, Leu73 of H2, and Leu277 of H8 were estimated to be potential barriers. Their increased contact frequency was attributed to their close proximity to a water access pathway or in the case of Trp104 of H3 to Ser105.

PWC was identified as the most frequented water access pathway (Fig. 4). Even residue Gln46, which was defined as the outer entrance in the study of Larsen [13], was resolved by SFM (Fig. 3). Water influx via PWC occurred in the loop region between $\alpha 5$ and $\beta 2$, according to the secondary structure numbering by Uppenberg [21]. Access to SWC occurred in the loop region between $\alpha 5$ and $\beta 5$, which is located in close proximity to the catalytically active Ser105 and is structurally slightly submerged relative to the protein surface, yet fully solvent accessible between $\alpha 7$ and the loop region between $\beta 6$ and $\beta 7$. Access to entrance IWE1, which is located directly at the boundary between CALB and the triglyceride layer,

occurred at the loop preceding $\alpha 9$, which was slightly denatured at its N-terminus due to the direct interaction with the triglyceride layer. IWE1 may thus contribute to the influx of water via the triglyceride layer and through the substrate channel, as water molecules were observed to "leak" in between the protein and the triglyceride layer (Fig. 5). Access to entrance IWE2 occurred in the loop region between $\beta 8$ and $\beta 9$ close to the C-terminus of CALB, which also directly borders the triglyceride-water interface and can thus potentially also contribute to the interfacial leakage.

8 The Solvent Flux Method in the Context of Hydrolase Engineering

Results from SFM suggest that the triglyceride layer, where the enzyme CALB is attached to in its catalytically active conformation, does not constitute a hydrophobic barrier that excludes water molecules from entering the enzyme via the buried substrate channel. On the contrary, significant interfacial leakage between the protein and the triglyceride interface was observed (Fig. 5) which is comparable in significance to the water influx via the known primary water channel (PWC) [13] and higher than the influx via the potential secondary water channel (SWC) identified by SFM (Fig. 4). The fact that during equilibration of the protein-triglyceride system the protein immersed into the triglyceride layer implies that the observed interfacial water leakage is not a consequence of incomplete protein adsorption in the model. The origin of the interfacial water leakage is instead accredited to the self-association structures of polar triglyceride moieties that are formed in aggregates at equilibrium [1]. Lamellar-like self-association structures might therefore serve as a polar microenvironment that can facilitate an influx of water molecules through the interface and into the active site via the substrate channel. In this regard, the polar amino acids of IWE1 and IWE2 are potential avenues for water access through the protein-triglyceride-water interface (Fig. 5). These considerations are subject of an ongoing study to clarify the involvement of triglyceride molecule association on interfacial water, where the quality of the interfacial model is placed under particular scrutiny. It was demonstrated that the solvent flux method presented in this study is able to successfully identify solvent access pathways in proteins, by revealing the primary water channel (PWC) and other key positions in CALB that may increase synthesis versus hydrolysis in non-aqueous media when appropriately mutated, as demonstrated by Larsen et al. [13]. Although SFM introduces artificial external forces to MD simulations by the periodic velocity adjustment of water molecules and may thus raise concerns about misleading biases, one must consider the limitations of conventional MD in sampling rare events such as the passage of a molecule through a channel, both in real and computational time. This is particularly difficult when seeking to simultaneously model multiple rare events. Due to the limitations of computational resources, exploring complex problems such as the solvent flux through an enzyme with significant statistics is currently not feasible by conventional MD approaches.

Therefore, increasing the sampling efficiency of MD by introducing biases to the potential function to overcome energy barriers has become a widely used strategy, such as accelerated MD (aMD) [8], replica-exchange MD (REMD) [24], steered MD (SMD) [15] or random acceleration MD (RAMD) [16]. The multitude of successful applications and insights attained by these methods is testament to their usefulness in studying biophysical effects that would otherwise be inaccessible. In this context, SFM offers the means to rapidly and comprehensively sample solvent flux behavior throughout a protein, which allows for an atomistic resolution of structural features and positions that are relevant to enzyme-solvent interactions. It is thereby unique in its approach of simultaneously modeling the accelerated influx of multiple solvent molecules that randomly come into contact with the protein from the solvent bulk, and to extract meaningful data in form of contact frequencies and compiled pathways of individual water molecules during influx. In this regard, other comparable methods are either less deliberate in overcoming specific energy barriers (aMD, REMD), are restricted to single molecules and predefined geometric reaction coordinates (SMD), or are more suited to resolving the efflux of single substrates (RAMD). SFM holds the potential of exploring solvent-enzyme interactions beyond the hydration-related context, which seems to be required, considering that identifying the role of residual water in facilitating enzyme activity in non-aqueous synthetic hydrolase applications has proven to be nontrivial [20]. Even studies on "dry" systems have shown that residual water is in fact not restricted to the hydration of proteins, but instead partitions into the solvent environment under equilibrium conditions [7]. Understanding the behavior of residual free water in reaction media may thus prove particularly useful for synthetic hydrolase applications in non-aqueous environments, specifically to eliminate water as a competing nucleophile. SFM in conjunction with the desired organic nucleophile may additionally benefit enzyme engineering in improving synthesis reactions by rational design. In principle, SFM can thereby aid in resolving the competition between synthesis and hydrolysis, which remains a fundamental problem in designing a particular hydrolase application and is difficult to overcome without a deeper mechanistic understanding of molecular solvent fluxes.

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