Enzyme Microheterogeneous Hydration and Stabilization in Supercritical Carbon Dioxide
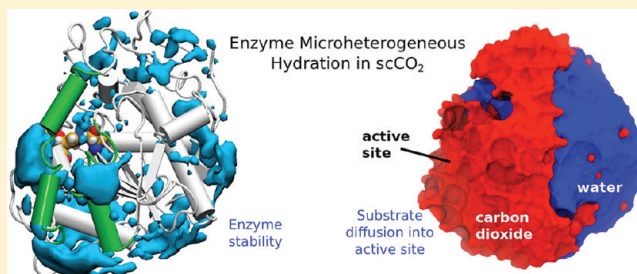
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S *Supporting Information*

ABSTRACT: Supercritical carbon dioxide is a promising green-chemistry solvent for many enzyme-catalyzed chemical reactions, yet the striking stability of some enzymes in such unconventional environments is not well understood. Here, we investigate the stabilization of the *Candida antarctica* Lipase B (CALB) in supercritical carbon dioxide—water biphasic systems using molecular dynamics simulations. The preservation of the enzyme structure and optimal activity depend on the presence of small amounts of water in the supercritical dispersing medium. When the protein is at least partially hydrated, water molecules bind to specific sites on the enzyme surface and prevent carbon dioxide from penetrating its catalytic core. Strikingly, water and supercritical carbon dioxide cover the protein surface quite heterogeneously. In the first solvation layer, the hydrophilic residues at the surface of the protein are able to pin down patches of water, whereas carbon dioxide solvates preferentially hydrophobic surface residues. In the outer solvation shells, water molecules tend to cluster predominantly on top of the larger water patches of the first solvation layer instead of spreading evenly around the remainder of the protein surface. For CALB, this exposes the substrate-binding region of the enzyme to carbon dioxide, possibly facilitating diffusion of nonpolar substrates into the catalytic funnel. Therefore, by means of microheterogeneous solvation, enhanced accessibility of hydrophobic substrates to the active site can be achieved, while preserving the functional structure of the enzyme. Our results provide a molecular picture on the nature of the stability of proteins in nonaqueous media.

1. INTRODUCTION

Supercritical carbon dioxide (scCO$_2$) is a promising solvent for green chemistry, as it is nontoxic, nonflammable, can be inexpensively produced by many industrial processes, and is able to solvate both hydrophobic and hydrophilic solutes.[1] Furthermore, solutes can be readily recovered from scCO$_2$ by depressurization. Synthetic chemistry in scCO$_2$ is, therefore, becoming increasingly important in industrial applications.[2]

Classical organic chemistry can be adapted to scCO$_2$ usually with the sole condition that reactants and products do not react directly with CO$_2$. Reactions can be performed with solid catalysts, for instance, by simply pumping reactants and products dissolved in scCO$_2$ through a porous support for the catalyst.[2] However, some reactions are hardly transferable to scCO$_2$ as some of their constituents either react with CO$_2$ or depend on the aqueous medium for activity. Among the latter, enzyme catalyzed reactions are most challenging since, although proteins may be stable in many uncommon media, virtually any protein evolved to preserve its fold in highly polar aqueous conditions, which hardly mimic scCO$_2$ environments.

Therefore, it is fascinating that naturally occurring (not engineered) enzymes are progressively being studied and used for catalysis of organic reactions in scCO$_2$, thus preserving their fold in scCO$_2$ to the point of maintaining, and even, increasing, their catalytic activity.[3,4] How can enzymes, or proteins in general, having evolved to function in aqueous solutions, preserve their structure and activity in such nonstandard environments is a question of fundamental importance for understanding the nature of protein folding, stability, solvation, and interactions, and has practical implications in protein design for green and sustainable chemistry.

Here we use molecular dynamics (MD) computer simulations to study molecular aspects of the stability of the *Candida antarctica* Lipase B (CALB) in scCO$_2$. This and similar lipases have been intensively used in the food and cosmetic industry for enantioselective hydrolysis of esters, and their stability in scCO$_2$ has been largely reported,[3−7] being one of the most promising enzymes for catalysis in scCO$_2$. As these enzymes catalyze the hydrolysis of triglycerides, which are hydrophobic substrates, the scale-up strategy for their application depends on their dispersion in nonpolar solvents.[2,5] However, scCO$_2$ has been shown to be a viable alternative which increases by almost 2 orders of magnitude the activity of lipases relative to hexane, for example.[8] Additionally, these enzymes increase their activity upon binding to oil−water interfaces, a remarkable property that is related to a large

hydrophobic surface surrounding the entrance of the catalytic funnel, although the active site itself contains hydrophilic residues and water molecules that are essential for the catalysis.[9]

Our results show that the CALB structure is preserved at the molecular scale in $scCO_2$−water systems but not in pure $scCO_2$. Water molecules are crucial for the stabilization of a rigid nucleus of the structure, whereas the exposure of the hydrophobic surface to CO_2 ensures functional flexibility. We also find that some water is essential for preserving the proper conformation of the catalytic triad, which involves an specific hydrogen-bonding arrangement between residues His224, Asp187, and Ser105.[9] The simulations indicate that CO_2 and water are heterogeneously distributed on the CALB surface in a way that may resemble enzyme binding to an oil−water interface. These results suggest that the microheterogeneous distribution of the cosolvents may facilitate access and binding of nonpolar substrates to the active site of the enzyme and provide a rational for understanding why these enzymes are most active in $scCO_2$ when some water is added to the system, as experimentally observed.[10] Microheterogeneous solvation may be a fundamental characteristic of the stabilization of proteins in nonaqueous media.

2. COMPUTATIONAL DETAILS

Molecular dynamics simulations were performed starting from a crystallographic structure of CALB (PDB ID: 1TCA),[9] after removing the carbohydrate bound to the enzyme. Considering the protonable residues in their most likely state in solution, the net charge of the protein is −1. For electrical neutrality, we mutated the glutamate residue at position 269 at the protein surface into glutamine.

Simulations were performed for CALB in $scCO_2$ with different water contents. Initial configurations were built with Packmol.[11] We adopted the OPLS-AA force field[12] for the protein, the TIP3P model[13] for water, and the elementary physical model EPM2 for CO_2.[14] The simulations were performed using rectangular periodic boundary conditions, in which the short-range interactions were truncated at a cutoff distance of 12 Å, whereas the electrostatic interactions were evaluated using particle mesh Ewald.[15] Covalent bonds involving hydrogen atoms were kept at their equilibrium lengths using RATTLE,[16] and a time step of 1.0 fs was used for integrating the equations of motion. Simulations in the supercritical state of CO_2 were performed by keeping the temperature at 328.15 K and pressure at 100 bar. The Langevin thermostat was used for temperature control and the Langevin piston for pressure control. Control simulations at 300 K and 1 bar were also performed.

Table 1 displays the characteristics of the simulated systems. In system 1, CALB is immersed is pure $scCO_2$. In systems 2−6, increasing quantities of water were added to fixed amounts of CO_2. The 286 water molecules of system 2 correspond to the crystallographic water of the 1TCA structure.[9] System 7 has a small amount of carbon dioxide in aqueous environment. In system 8, the enzyme is solvated by pure water and simulated at 300 K and 1 bar. In system 9, the enzyme is immersed with pure water at 328.15 K and 100 bar.

In order to decide the best way of distributing the less abundant cosolvent in the simulation box, we first performed an MD simulation of system 6 with both solvents homogeneously distributed around CALB in the simulation box as starting configuration (Figure 1A). The result of this preliminary simulation was that the water molecules readily formed

Table 1. Details of the Simulations

system	notation[a]	# water molecules	# CO_2 molecules	temperature (K)	pressure (bar)
1	$N_w = 0$	0	10 000	328.15	100
2	$N_w = 286$	286	10 000	328.15	100
3	$N_w = 500$	500	10 000	328.15	100
4	$N_w = 1000$	1000	10 000	328.15	100
5	$N_w = 2000$	2000	10 000	328.15	100
6	$N_w = 3000$	3000	10 000	328.15	100
7	$N_w = \infty*$	15 292	1000	328.15	100
8	$N_w = \infty**$	15 292	0	300	1
9	$N_w = \infty***$	15 292	0	328.15	100

[a]The symbol $\infty*$ refers to the enzyme immersed in water plus 1000 CO_2 molecules, $\infty**$ refers to the enzyme in pure water at room conditions (300 K and 1 bar), and $\infty***$ to the enzyme in pure water at 328.15 K and 100 bar.
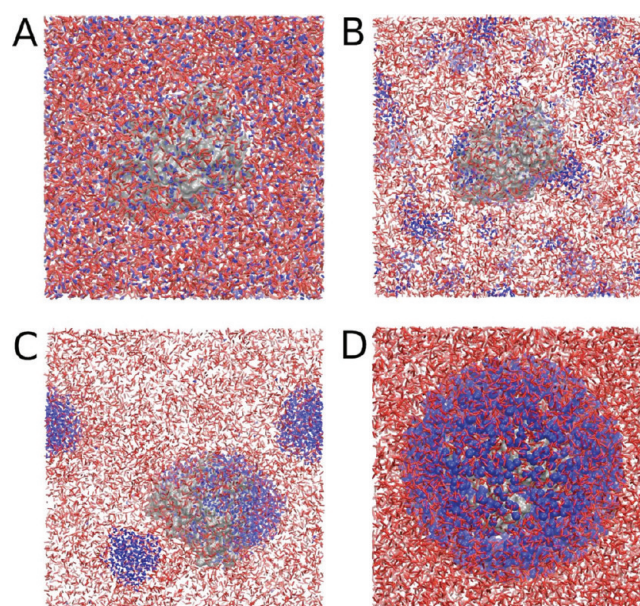

Figure 1. (A) Initial configuration with water (blue) and CO_2 (red) molecules homogeneously distributed in the simulation box. The protein is at the center. (B) Snapshot after 5 ns, showing several clusters of water molecules. (C) Snapshot after 10 ns, showing that the clusters are captured by the protein surface. (D) An initial configuration showing a water shell around the protein surface. The figures are not drawn in the same scale, since the initial volume of the system varied considerably to reach the correct density at the conditions of the simulations.

aggregates in the CO_2 medium (Figure 1B). These aggregates then accumulated on the protein surface (Figure 1C). In light of this result, we generated initial configurations for all other systems such that the less abundant solvent forms a spherical shell around the enzyme (Figure 1D).

All simulations were carried out at thermodynamic conditions well below the experimental or TIP3P water critical point.[17] Under these conditions, our simulations are reasonably far from the experimental or computer simulated boundary of stability (miscibility) of water and CO_2, as reported by Vorholz et al.[18] for the EPM2 potential and several water models. Therefore, our solvent environments are not supercritical homogeneous solutions of CO_2 and water, but are, instead,
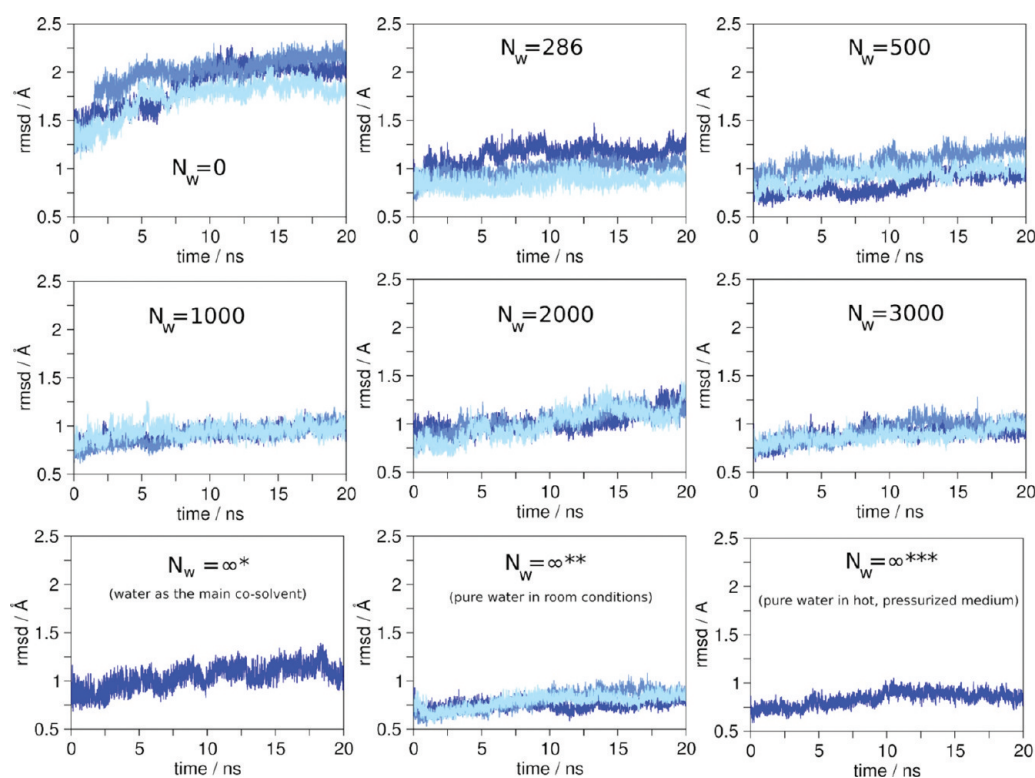
Figure 2. rmsd of the α-carbons corresponding to the 70% less flexible residues of the enzyme (r-rmsd) relative to the crystallographic structure. In pure scCO$_2$, the structural core of the enzyme is not preserved. The structure is preserved only in the presence of water. However, stability is largely insensitive to the amount of water molecules available in the system (N_w). The three different curves of each system correspond to the three independent simulations. The symbols ∞^*, ∞^{**}, and ∞^{***} are defined in Table 1.

interfacial biphasic systems consisting of liquid water and supercritical CO$_2$. Our CO$_2$/water simulation conditions are not far from those employed by da Rocha et al.[19] in their study of a liquid water−supercritical CO$_2$ interface.

Three independent 20 ns long simulations were performed for systems 2−6 and 8 for improved sampling. Single 20 ns runs were performed for control systems 7 and 9. All runs were performed with NAMD.[20] Analyses were performed with VMD[21] and in-house programs. Before production runs, the systems were subject to the following equilibration steps: (a) 2000 steps of minimization of energy using conjugate gradients (CG) followed by 200 ps MD with all of the atoms of the enzyme fixed, (b) 1000 CG steps followed by 200 ps MD keeping the whole system free, except the α-carbons of the protein, and (c) 600 ps of MD without any restraints. Equilibrium properties were computed from the last 8 ns of the 20 ns production runs.

The structural fluctuations of the enzyme were probed by the root-mean-square deviations (rmsd) using a robust alignment algorithm.[22−24] The procedure consists of the following steps: (1) alignment (standard rigid-body rmsd minimization) of all α-carbons, (2) computation of the deviation of each α-carbon from a reference structure, (3) selection of the N residues that presented the lowest deviations, (4) new alignment considering only the residues selected in step 3, and (5) repetition of steps 2−4 until the identity of the N residues remains invariant. This way, the residues located in the more rigid regions of the protein dominate the alignment. Thus, the mobility of the more flexible residues will be highlighted, giving a picture of the behavior of the mobile regions relative to the less flexible parts of the protein. After such alignment, two rmsds were

computed: (a) rigid-fraction-rmsd (r-rmsd), which considers only the N residues used for the alignment, and (b) flexible-fraction-rmsd (f-rmsd), which is computed for residues not used for the alignment. Here, we chose N such that it corresponded to ~70% of the residues, i.e., $N = 230$. The actual identity of the residues follows automatically from the algorithm without intervention. Therefore, r-rmsd refers to the 70% less mobile residues and portrays the rigid portions of the enzyme, whereas f-rmsd refers to the 30% most mobile residues and captures the most flexible parts of the structure.

3. RESULTS AND DISCUSSION

3.1. Enzyme Stability and Active Site Conformation in scCO$_2$−Water Systems. The stability of the CALB structure in different scCO$_2$−water environments is dependent on the presence of water. According to the results shown in Figure 2, the r-rmsd values in the absence of any water increase sharply and surpass 2.0 Å during the simulated time, indicating that the protein rigid core in pure scCO$_2$ deviates significantly from the crystallographic structure. In the presence of water, however, the enzyme remains stable and shows no significant distortion in its more rigid region, as the r-RMSDs oscillate around 1.0 Å or so. No marked differences in the global behavior of the enzyme are observed with increasing amount of water. Very similar conformational deviations are observed for all systems containing water. That is, from the enzyme in scCO$_2$ in the presence of crystallographic water ($N_w = 286$) to the enzyme in pure water at ambient or in hot pressurized conditions. Hence, according to the simulations, scCO$_2$ alone is not able to stabilize the native enzyme structure. The native structure of the enzyme in scCO$_2$ depends on the presence of traces of
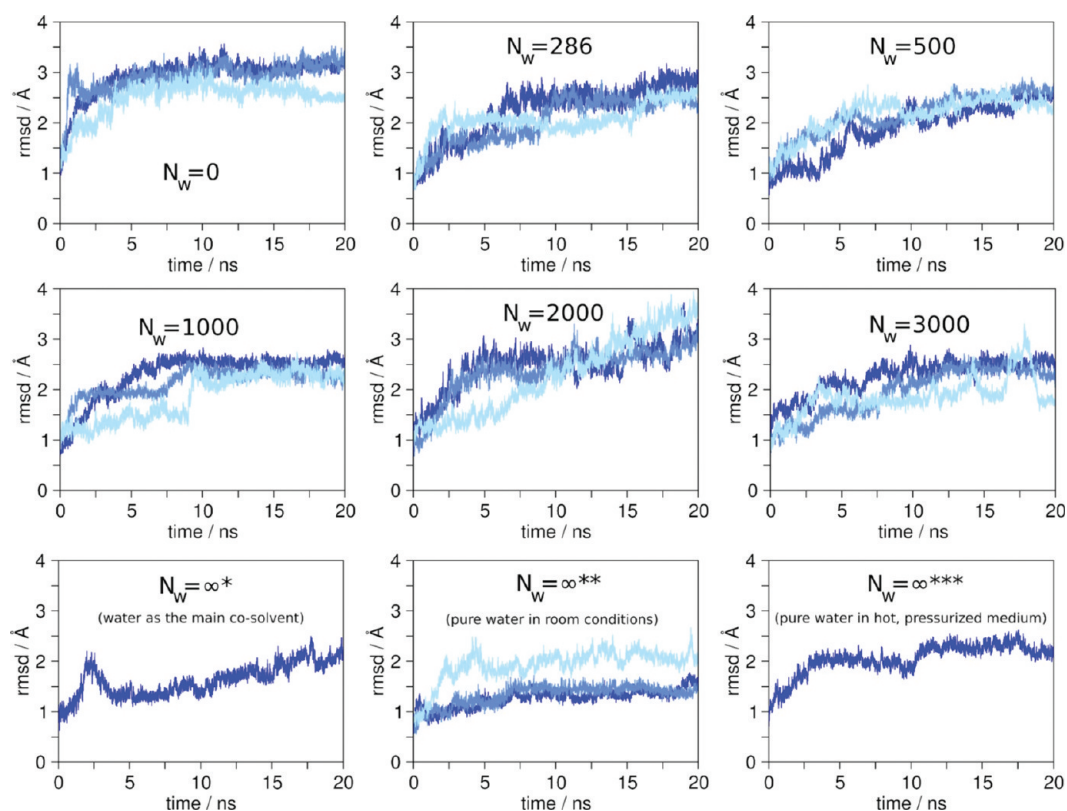
Figure 3. rmsd of the α-carbons corresponding to the 30% more flexible residues of the enzyme (f-rmsd) relative to the crystallographic structure. The flexible parts of the enzyme exhibit pretty much the same behavior in the systems in which the $scCO_2$ is the main solvent. When the water is the main solvent, a subtle stabilization is observed. N_w refers to the number of water molecules present in the system. The symbols $\infty*$, $\infty**$, and $\infty***$ are defined in Table 1.

water, which are sufficient for preserving the structure of the protein rigid core. This is consistent with previous experimental results showing that CALB has no enzymatic activity in dry $scCO_2$ condition.[10]

The more mobile regions of the enzyme (~30% of the residues) exhibit relatively large structural fluctuations in every environment considered. As illustrated in Figure 3, structural deviations are similar for all simulations in which the main solvent is $scCO_2$, including those corresponding to the protein in dry $scCO_2$. In these cases, the f-RMSDs reach 2.0–3.0 Å or more by the end of the simulations. When water is the main cosolvent (systems 7–9, $N_w = \infty*$, $\infty**$, or $\infty***$), the mobility of the flexible region is somewhat smaller, reaching about 2.0–2.5 Å at most. These results indicate that $scCO_2$ increases slightly the mobility of the more flexible parts of the enzyme when compared to water. At the same time, it is noteworthy that the portions of the protein that are highly mobile in wet-$scCO_2$ systems are also mobile in pure water. This suggests that in these biphasic environments the protein is able to preserve both the structure of its rigid core and the flexibility of regions for which the mobility may be functionally relevant.

To illustrate this point, Figure 4 depicts the global mobility over CALB three-dimensional structure under three representative conditions: pure $scCO_2$, pure ambient water ($N_w = \infty**$), and in $scCO_2$ with 1000 water molecules (system 4). Pronounced conformational variations are clearly seen for the enzyme in pure $scCO_2$ in comparison to that in pure water or with a thin water shell. The front view, highlighting the entrance to the active site pocket, reveals that large-amplitude

structural fluctuations occur in the vicinity of the 140–146 region (helix $\alpha5$) at the top the funnel entry, and in the long helix $\alpha10$ at the C-terminus (region 268–287). The nomenclature of the secondary structure follows that proposed by Uppenberg et al.[9] The region illustrated in the back view is markedly more stable and it is significantly perturbed only in pure $scCO_2$. There is great similarity of enzyme motions in pure water or surrounded by a thin water shell.

The active site of CALB consists of the catalytic triad formed by residues Ser105, Asp187, and His224, the same sequential order of all known lipases.[9] In the active conformation, His224 is a hydrogen (H)-bond acceptor to Ser105 and a donor to Asp187, as shown in Figure 5. Recent MD simulations of CALB in different organic solvents have indicated that the H224-Asp187 H-bond is preserved in polar and nonpolar solvents, whereas the mean distance between Ser105 and H224 residues suggests that the H-bonding between these residues is, on the average, disrupted in polar solvents such as methanol, acetone, and tetrahydrofuran.[25] Here, we have computed the Ser105-H224 (d1) and H224-Asp187 (d2) distances from snapshots taken along the trajectories for CALB in pure $scCO_2$ without crystallographic waters (system 1, cf. Table 1), in $scCO_2$ with $N_w = 286$ crystallographic water molecules (system 2) and with $N_w = 1000$ (system 4), and in pure ambient water ($N_w = \infty**$, system 8). The results show that the active conformation of the catalytic triad is severely disrupted in the complete absence of water and preserved when some water is present. In addition, Figure 5 shows that the spatial arrangement of the triad is very similar whether the protein is in pure water or in $scCO_2$-water systems with different amounts of water.
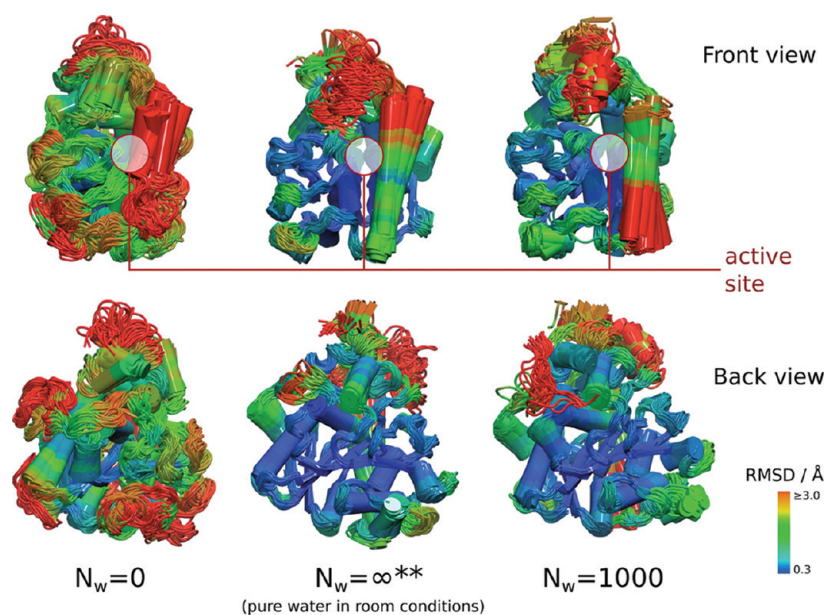
Figure 4. Structural stability of CALB in pure dry $scCO_2$ (left), in pure water at room conditions (center), and in sCO_2 in presence of 1000 water molecules (right). Several superposed trajectory snapshots are shown. Ranging from blue to red, the color scale represents the rmsd of the α-carbon of each residue. The rmsds were computed relative to the average structure after the structural alignment of the structures. It is observed that the region around the active site is the most flexible and that $scCO_2$ perturbs substantially the less flexible regions of the enzyme.
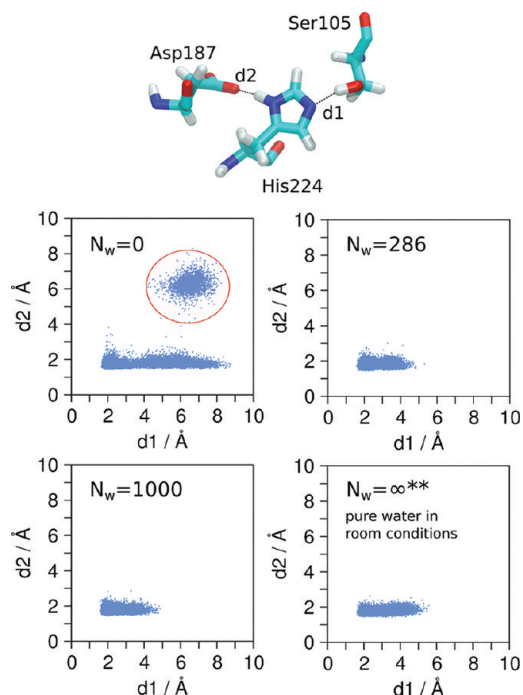

Figure 5. Conformational stability of the catalytic triad: In pure $scCO_2$ (N_w = 0) the catalytic triad assumes conformations that differ markedly from the active ones, as highlighted by the red ellipses. In pure water at room conditions (N_w = ∞**) or in $scCO_2$ with different amounts of water (N_w = 286 and 1000), the relative dispositions of these residues are similar and oscillate moderately in the vicinity of the active conformation, in which two hydrogen bonds are formed.

Figure 6. Average solvation per residue in the first solvation shell of the 150 hydrophobic and 167 hydrophilic residues of the enzyme by (A) $scCO_2$ and by (B) water, as function of the water content (N_w). The symbols ∞* and ∞** are defined in Table 1. The solvation level is computed as the number of cosolvent molecules within a 4.0 Å radius from any atom of the protein divided by the number of hydrophobic or hydrophilic residues. Only the last 8.0 ns of the trajectories were used in this analysis. The error bars refer to the ensemble fluctuations.

3.2. Microheterogeneous Solvation and Stability. The solvent distribution on the protein surface is strikingly heterogeneous due differential solvation of hydrophilic and hydrophobic residues. In Figure 6, we display the average
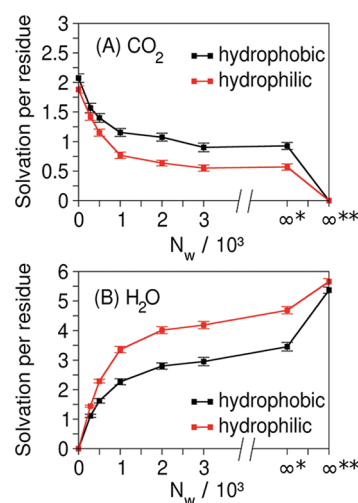
solvation of hydrophilic and hydrophobic amino acid residues by water and CO_2. A total of 150 hydrophobic and 167 hydrophilic residues were considered in the analysis. The data show, not unexpectedly, that hydrophobic residues are mostly solvated by CO_2, whereas hydrophilic ones are preferentially hydrated. As the amount of water increases, average solvation by CO_2 of both hydrophilic and hydrophobic residues decreases. This reflects water's ability to displace CO_2 molecules from the protein surface. However, the data also

indicate that, no matter the amount of water present, the first CO_2 solvation shell persists. Even in the water saturated regime (system 7, labeled ∞* in Figure 6), there is a substantial fraction of the protein surface covered by CO_2 molecules, at an average of 1 and 0.75 CO_2 molecules per hydrophobic and hydrophilic residues, respectively. Hence, there is no possibility for water to shield completely the protein surface from the $scCO_2$ medium and prevent protein−CO_2 contacts. Thus, the simulations show that microheterogeneous solvation leads to the preservation of the enzyme structure in water−$scCO_2$ systems.

As already described, the CALB structure is not preserved in dry $scCO_2$. The reason for this behavior is a continuous penetration of CO_2 molecules into the core of the enzyme, which is facilitated by a number of conformational changes involving two α-helical regions, which are represented in green in Figure 7A (these are the long and highly hydrophobic helix
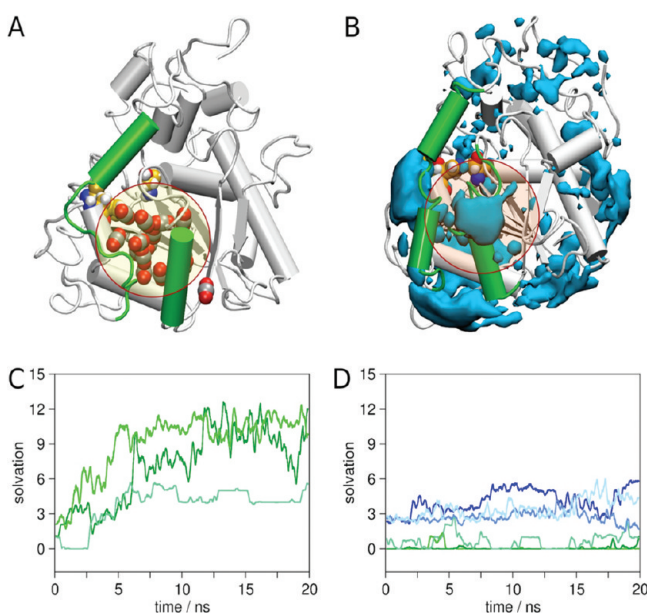

Figure 7. (A) In pure $scCO_2$, protein conformational alterations (green) allow CO_2 to invade the core of the enzyme. (B) When water is present, the region that would be accessible to $scCO_2$ is blocked, as shown by the water density map around the enzyme, highlighted in red. (C) Time evolution of CO_2 solvation of residues located in the core of the enzyme in dry conditions. (D) Time evolution of CO_2 (green) and water (blue) solvation when at least the crystallographic waters are present. Solvation levels were computed based on the number of cosolvent molecules within 5 Å from any of the residues Trp52, Phe131, or Leu228, at the protein core.

α10 and the α-helical region comprising residues 44−57).[9] Figure 7C shows the time evolution of the number of CO_2 molecules that reach within 5 Å from residues Trp52, Phe131, or Leu228 located at the core of the enzyme during the three independent simulations in pure $scCO_2$. Owing to its ability to solvate hydrophobic and hydrophilic residues, mainly in absence of water, CO_2 is able to readily reach the hydrophobic core of the protein and perturb the overall CALB fold. Under these conditions there is a clear disruption of the relative positions of the amino acid residues that form the catalytic triad, as already shown in Figure 5.

In contrast, when water is present, the core of the enzyme is protected from CO_2 molecules. Water molecules accumulate around specific sites on the enzyme surface, as shown by the

density map in Figure 7B. In particular, one of the regions occupied by water molecules comprises the channel through which CO_2 molecules is seen to diffuse into the protein core in the absence of any water (highlighted red). As shown in Figure 7D, some water molecules are found at the entrance this channel (shown by the blue curves and the density map) and impair CO_2 molecules (green lines) from breaching in. Thus, by blocking specific surface sites and impairing CO_2 penetration into the core of the protein, water prevents enzyme deactivation. Since CO_2 penetration was found to occur only through some specific region of the protein surface, small quantities of water should suffice to impair CO_2-induced disruption of the enzyme active structure.

These results provide a physically sensible qualitative explanation for the experimentally observed reversible activation of the enzyme in $scCO_2$ by addition of water.[10] According to the simulations, we would not expect to observe full denaturation of CALB in pure $scCO_2$, and the addition of water should readily restore its active conformation in $scCO_2$-water biphasic systems. Too much water, however, is known to reduce activity of lipases.[2,9,10] The simulations indicate that the preservation of the native structure of the enzyme is insensitive to the amount of water present in the system, but that in pure water the mobility of flexible parts of the protein are somewhat reduced. Therefore, water does not seem to induce enzyme denaturation under the conditions considered. The inactivation of the enzyme at high water activities might be due to the lack of flexibility of functionally mobile regions, or extrinsic effects such enzyme aggregation, competitive inhibition by water or decreased diffusion of the substrates, as previously suggested.[10]

3.3. Heterogeneous Microenvironment Suitable for Substrate Diffusion. One of the main advantages of using $scCO_2$ as a reaction medium for lipases enzymatic catalysis lies in its gas-like transport properties while retaining a liquid-like solvation ability. For reactions involving hydrophobic compounds such as lipids, their limited solubility is often a drawback for carrying out enzymatic catalysis in aqueous media. Therefore, the use of $scCO_2$ is an excellent alternative because in such environments the substrate can readily find its way into the active site of the enzyme by diffusion and the reaction products easily released and recovered. Our simulations indicate that the microenvironment created by $scCO_2$ and water around CALB may favor such processes. When there is enough water to form several solvation layers around the enzyme in a $scCO_2$ medium, water is not evenly spread over the enzyme surface, but instead accumulates around the more hydrophilic hemisphere at the opposite side of the active site. As a result, two well-defined solvation caps appear at the surface of the protein, such that the surface region surrounding the entrance to the deep catalytic funnel active-site region is exposed to CO_2, as shown in Figure 8. This is appealing, since this microenvironment around the enzyme is suitable for the diffusion of hydrophobic compounds, which may favor the lipase activity by mimicking its functionality in oil−water interfacial systems.

3.4. Role on CO_2 on Enzyme Structure and Mobility. The fact that CALB requires only a small amount of water to be stabilized in $scCO_2$ raises the question whether $scCO_2$ induces destabilization of the enzyme in the absence of water or the destabilization is just a consequence of the lack of structural water molecules. Experimentally, CALB is not active in dry $scCO_2$, but activation can be readily recovered by adding small quantities of water.[10] Therefore, one should not expect full
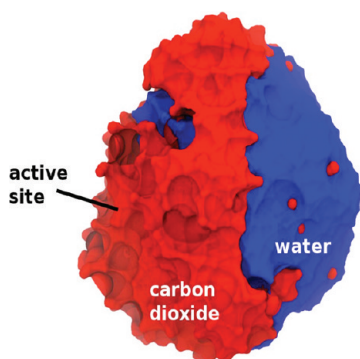
Figure 8. Molecular surface of the solvent around the enzyme showing a heterogeneous distribution of water and carbon dioxide at the end of the simulation with $N_w = 3000$. The more hydrophobic region of the active site is mainly solvated by CO_2, whereas the region across the active site is mainly solvated by water. The surface was built considering all solvent molecules within 10 Å from the protein surface.

denaturation of CALB in pure $scCO_2$. Our control simulation, in which we see water diffusing rapidly from a homogeneous distribution to the hydrophilic protein surface (Figure 1) is suggestive of the mechanisms underlying the fast reactivation of the enzyme by the addition of water. Two additional control simulations were performed to probe the relative importance of water and $scCO_2$ to CALB structure. First, a simulation was performed in pure water starting from the 1TCA structure striped of all crystallographic water molecules. No significant structural alterations were observed relative to the simulations with hydration plus crystallographic water molecules (Figure S1, Supporting Information). Thus, the overall stability of CALB is more dependent on the aqueous solvation of its hydrophilic surface than on a few inner-core water molecules. Evidently, some of the crystallographic positions can be, and were, readily reoccupied by water coming from the bulk. Second, a simulation of CALB plus its crystallographic water molecules was performed in vacuum (Figure S2, Supporting Information). The structure in vacuum is more rigid than in $scCO_2$, resembling the simulation in pure water. Therefore, $scCO_2$ increases the mobility of the protein and this is likely to facilitate substrate diffusion into the enzyme.

4. CONCLUDING REMARKS

The folding of proteins into their functional states is a ubiquitous phenomenon in living matter, where water is omnipresent. In fact, it is widely accepted that proteins have evolved to acquire their native functional states in aqueous environments. Nevertheless, the reversible unfolding/refolding of proteins has been demonstrated to occur in nonaqueous media such as ionic liquids,[26,27] organic solvents, and supercritical carbon dioxide.

The structural stability and activity of certain enzymes in $scCO_2$ under different moisture conditions is a remarkable fact not only because of its importance for practical green chemistry applications, but also because of its inherent scientific relevance. Although the phenomenon has been observed for quite some time, little has been known hitherto about how the enzyme maintains its native active fold in mixed $scCO_2$–water environments. It is generally believed, without further justification, that a hydration layer is formed all around the protein surface providing an aqueous microenvironment within

which the protein fold is preserved, whereas CO_2 acts merely as a dispersing medium.

In this work we investigate the stability of the active conformation of a lipophilic enzyme (*Candida antarctica* Lipase B) immersed in $scCO_2$-water biphasic systems with varying water content using molecular dynamics simulations. Contrary to common belief, our simulations show that water and CO_2 molecules solvate the protein surface in a highly heterogeneous fashion. Water molecules bind to specific sites on the protein surface and prevent CO_2 molecules from invading the catalytic core of the protein, which is the main cause of the disruption of the enzyme's active conformation under dry $scCO_2$ conditions. Our results also indicate that by means of heterogeneous solvation CO_2 molecules accumulate preponderantly at the entrance of the catalytic funnel of lipase B, even at high water activities, conferring the necessary protein mobility for an optimal capture of hydrophobic substrates that these enzymes target. All together, the simulations suggest that the micro-heterogeneous solvation of the protein surface is essential for CALB optimal activity and that CO_2 plays a role to the enzyme's activity beyond that of being just a dispersive medium. Microheterogeneous solvation may be a fundamental principle behind the stability of proteins in supercritical solvents or other nonconventional media.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

α-carbon rmsd for CALB in water, with and without crystallographic water molecules, and in vacuum and in pure $scCO_2$ with crystallographic water molecules. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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