Structural determinants of ligand imprinting: A molecular dynamics simulation study of subtilisin in aqueous and apolar solvents

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Received 2 October 2010; Accepted 19 November 2010

DOI: 10.1002/pro.569

Published online 8 December 2010 proteinscience.org

Abstract: The phenomenon known as “ligand imprinting” or “ligand-induced enzyme memory” was first reported in 1988, when Russell and Klibanov observed that lyophilizing subtilisin in the presence of competitive inhibitors (that were subsequently removed) could significantly enhance its activity in an apolar solvent. (Russell and Klibanov, *J Biol Chem* 1988;263:11624–11626). They further observed that this enhancement did not occur when similar assays were carried out in water. Herein, we shed light on the molecular determinants of ligand imprinting using a molecular dynamics (MD) approach. To simulate the effect of placing an enzyme in the presence of a ligand before its lyophilization, an inhibitor was docked in the active site of subtilisin and 20 ns MD simulations in water were performed. The ligand was then removed and the resulting structure was used for subsequent MD runs using hexane and water as solvents. As a control, the same simulation setup was applied using the structure of subtilisin in the absence of the inhibitor. We observed that the ligand maintains the active site in an open conformation and that this configuration is retained after the removal of the inhibitor, when the simulations are carried out in hexane. In agreement with experimental findings, the structural configuration induced by the ligand is lost when the simulations take place in water. Our analysis of fluctuations indicates that this behavior is a result of the decreased flexibility displayed by enzymes in an apolar solvent, relatively to the aqueous situation.

Keywords: ligand imprinting; enzyme memory; enzymes in nonaqueous solvents; catalysis; subtilisin; MD simulation

Introduction

Enzymatic catalysis in anhydrous solvents has attracted the attention of biotechnologists and biochemists for more than two decades. In nonaqueous media, enzymes can display several novel and valua-

ble properties,¹ such as the capacity to catalyze reactions that are not feasible in water,² interfacial activation,^{3,4} increased thermostability,⁵ and an altered substrate specificity as well as enantiomeric selectivity.^{6–9} It is now clear that reactions in nonaqueous media are strongly dependent on the water content of the solvent. The amount of water can influence enzymatic properties like activity,¹⁰ structure,^{11–13} dynamics,^{11–13} and enantioselectivity,^{8,12} and can thus be used to control the catalytic process. Despite their great potential, reactions in nonaqueous solvents are often limited by a drastic reduction in enzyme activity when compared with their aqueous counterparts.¹⁴ This raises an obvious question: How

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Fundação para a Ciência e a Tecnologia, Portugal; Grant numbers: SFRH/BD/28269/2006 and POCTI/BIO/57193/2004.

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can the activity of enzymes in organic solvents be enhanced?

In 1988, Russell and Klibanov observed that the enzymatic activity of the serine protease subtilisin, in anhydrous *n*-octane, could be enhanced by previously lyophilizing the enzyme in the presence of competitive inhibitors.¹⁵ In their study, the ability of five different inhibitors to enhance the rate of transesterification reactions was tested. The authors reported an increase of up to ~100 fold in enzyme activity relatively to the enzyme lyophilized in the absence of inhibitors. This was the first description of a curious phenomenon known as “ligand-induced enzyme memory” or “ligand imprinting.” Interestingly, when the same assays were carried out in water, there was no difference between the enzyme preparations lyophilized in the presence and in the absence of inhibitors, indicating that the enzyme loses its “memory” in water. Moreover, the authors found a clear correlation between the percentage of water retained in the organic solvent and the observed rate enhancement: the larger the water content, the smaller the rate enhancement. In an attempt to explain this behavior, they speculated that the competitive inhibitor causes a conformational change in the enzyme that is retained in anhydrous apolar solvents, even after the removal of the ligand, because the enzyme is rigid in the absence of water and thus it gets kinetically trapped in the conformation induced by the inhibitor: the enzyme behaves as if it has a “memory.” As the water content increases, the protein becomes more flexible and rapidly “forgets the ligand imprinted state.”

In another study, Staahl *et al.* showed that the substrate specificity and stereoselectivity of α -chymotrypsin in anhydrous organic media could be tuned by using an enzyme preparation obtained by precipitation with different inhibitors.¹⁶ These results show that the activation increases as the similarity between the substrate and the inhibitor used for “imprinting” increases, indicating that the effect is very specific and located in the active site.

The application of molecular imprinting has been extended by Rich and Dordick to the activation of subtilisin-catalyzed acylation of nucleosides.¹⁷ The authors complemented their experimental findings with a molecular dynamics study and concluded that the activation of enzymes by imprinting is caused by structural changes of the catalytic triad.

The molecular determinants of the observations reported above remain unclear. In this work, we addressed this question by mimicking the effect of lyophilizing subtilisin in the presence and in the absence of an inhibitor and then performing MD simulations using the resulting structures, both in hexane and in water. Our results indicate that the inhibitor induces an open conformation of the S1

pocket that is maintained after the removal of the ligand in anhydrous, but not in aqueous, simulations. Our analysis of fluctuations suggests that this behavior is caused by the decreased flexibility exhibited by subtilisin in hexane.

Results and Discussion

The hypothesis analyzed in this study is that a ligand in complex with an enzyme induces conformational changes in the active site that can be maintained once the ligand is removed and the protein is immersed in an anhydrous apolar solvent. However, if the protein is immersed in water, its conformation rapidly deviates from the ligand-induced one.

To test this hypothesis, we used the strategy summarized in the fluxogram represented in Figure 1. As the fluxogram shows, we performed two distinct sets of simulations, the first set will be referred to as “ligand-treated” simulations and the second set will be called “ligand-untreated” simulations.

In the ligand-treated simulations, we started by docking an inhibitor in the active site of subtilisin. We then placed the enzyme-ligand complex in water and performed 30 independent MD simulations of 20 ns each. The purpose of these simulations was to adapt the active site to the ligand. In 16 out of the 30 simulations carried out the ligand remained in the catalytic pocket and the final structures of these 16 simulations were used in the subsequent steps of the methodology. The next step consisted in the removal of the inhibitor. Finally, we conducted 10 ns of MD in *n*-hexane (which is similar to *n*-octane) and in water, using as a starting point the conformations obtained in the previous step.

As a control, we performed a set of 16 ligand-untreated simulations, in which we began by running 20 ns of MD simulations in water, starting from the x-ray structure of subtilisin. We then used the final conformations of these simulations to carry out 10 ns of MD in hexane and in water.

Docking of the inhibitor

Given that the S1 pocket, which is the specificity subsite in serine proteases, is known to accommodate hydrophobic substrates¹⁸ (as it is the case for our inhibitor), we restricted our docking searches to the area surrounding this site. The best docking solution found is displayed in Figure 2.

This solution was reached in 116 out of 500 runs and has the lowest docked energy of all the solutions found. As can be seen in the figure, the ligand is docked very near the catalytic triad, preventing substrates from binding. Therefore, this docking position is compatible with the competitive character of the inhibition of subtilisin by *N*-acetyl-L-tryptophan amide. These results, led us to choose the enzyme-ligand complex shown in Figure 2 as the

Figure 1. Overview of the simulation methodology.

starting point for all the subsequent steps of this study.

Stability of the simulations

The temporal evolution of the root mean square (r.m.s.) deviation from the x-ray structure provides information on the stability of a simulation. Supporting Information Figure S2 shows the r.m.s. deviation from the crystal structure, for the final 10 ns of all the systems studied (see Fig. 1). The plots show that in general the simulations carried out in hexane [Supporting Information Fig. S2(A) and S2(B)] stabilized slower than the simulations that were performed in water [Supporting Information Fig. S2(C) and S2(D)]. This observation can be explained by the

fact that the starting points of these simulations are the final conformations obtained after 20 ns of simulations in water (see Fig. 1). In the case of hexane simulations, the protein is placed in a new medium and the system has to reach a new equilibrium state. However, in the simulations carried out in water, the protein is kept in the same environment and there is no adaptation phase. Looking at the plots in Supporting Information Figure S2, we can also observe that in hexane simulations the protein deviates more from the crystal structure than in water simulations, which probably reflects the fact that hexane is an unnatural medium for proteins, which leads them to adopt a different conformational arrangement. In the great majority of the

Figure 2. Stereo view of the best docking solution of the inhibitor *N*-acetyl-L-tryptophan amide in the S1 site of subtilisin. The atoms of the ligand are represented using spheres. The residues that compose catalytic triad are represented with sticks and labeled. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 3. Illustration of the three distinct states adopted by the S1 pocket. (A) Example of a closed conformation (last configuration of replicate number 3 of the ligand-untreated simulations in hexane). (B) Example of an intermediate conformation (last configuration of replicate number 4 of the ligand-untreated simulations in water). (C) Example of an open conformation (last configuration of replicate 3 of the ligand-treated simulations in hexane). (D) The minimum distance between the two loops (represented by a red arrow) can be used to analyze the state of the pocket. This measurement corresponds to the minimum distance between all the atoms of residues 125 to 127 and residues 153 to 155. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

simulations, the r.m.s. deviation stabilizes before 5 ns of simulation time; thus we considered that the MD simulations were equilibrated after that period.

Effect of pretreating the enzyme with the ligand: hexane vs. water simulations

As mentioned above, the ligand was docked in the S1 pocket. It is therefore relevant to analyze the behavior of this pocket after the removal of the ligand and compare this behavior with the ligand-untreated case. From the analysis of our simulations we observed that the S1 pocket can adopt three different states, that we named “closed,” “intermediate,” and “open.” These states are illustrated in Figure 3.

To have a quantitative measurement that could capture the state of the S1 pocket during the simulations, we calculated the minimum distance between the two loops surrounding the pocket (see the illustration in Figure 3D). The histograms in Figure 4 represent the distributions of these distances in the last 5 ns of simulation (when the simulations were considered equilibrated). In Figure 4(a) we can see that the distributions of the ligand-treated and the

ligand-untreated simulations in hexane are clearly distinct, which indicates that the inhibitor has an effect on the structural arrangement of the S1 pocket. The distribution of the ligand-treated simulations is more extended and there is a considerably larger number of conformations with an open pocket and a smaller number of conformations presenting a closed or intermediate pocket.

The plots provided in Supporting Information Figure S3(A) indicate that the inhibitor induces an open state of the S1 pocket that in many cases is retained when the enzyme is placed in hexane after the removal of the ligand. This behavior is illustrated in the movie S4 (Supporting Information).

When the enzyme has no contact with the ligand, the pocket tends to have a more closed configuration that is maintained or even accentuated when the enzyme is placed in hexane [Supporting Information Fig. S3(B)]. As a consequence, pretreating the enzyme with a competitive inhibitor

Figure 4. Distributions of the minimum distance between the loops surrounding the S1 pocket during the last 5 ns of simulation. The three distinct areas highlighted in the plots, in different tones of gray, correspond to three different states of the pocket (closed, intermediate, and open, respectively). (A) Ligand-treated (solid line) and ligand-untreated (dashed line) simulations in hexane. (B) Ligand-treated (solid line) and ligand-untreated (dashed line) simulations in water.

increases the probability of finding an open S1 pocket.

The broad distribution exhibited by ligand-treated simulations [Fig. 4(A)] can be explained by the fact that during the accommodation step, when the enzyme-ligand complex is simulated in water, the ligand adopts several distinct conformations, which influence the structure of the active site. Therefore, at the beginning of the ligand treated simulations, each replicate displays a different conformation of the active site, which tends to be retained in hexane, giving rise to the extend distribution shown in the plot.

In opposition to what was described for hexane, in water, the distributions of the ligand-treated and ligand-untreated simulations are very similar [Fig. 4(B)]. The plots in Supporting Information Figure S3(C) show that when the enzyme is kept in water after the removal of the ligand, the S1 pocket tends to deviate from the open state induced by the ligand and reach an intermediate state, in which the loops are separated by a distance between 0.4 and 0.5 nm. The behavior of the S1 pocket in the ligand-treated simulations in water is illustrated in the movie S5 (Supporting Information).

When the enzyme is placed in water with no a priori contact with the inhibitor, the pocket remains in the same intermediate conformation (Supporting Information Fig. S3(D)). The homogeneity between the ligand-treated and control simulations indicates that in water the ligand has no imprinting effect.

These observations give an insight into the molecular determinants that are on the basis of the experimental findings made by Russell and Klibanov.¹⁵ Our results indicate that, in hexane, the active site of subtilisin tends to be more open when the enzyme is pretreated with a competitive inhibitor. It is easier for a substrate to bind to an open active site and this explains the fact that the enzymatic activity, towards substrates that are similar to the inhibitor, increases when the enzyme is lyophilized from a solution containing a competitive inhibitor. In water, when subtilisin is pretreated with an inhibitor and then washed, the pocket tends to rapidly deviate from the conformation induced by the ligand and adopt a configuration that is similar to the one found in enzyme molecules that had no contact with the ligand. This accounts for the lack of rate enhancement observed when the reactions were performed in water, after placing the enzyme in the presence of a competitive inhibitor and then removing the ligand.

Why does ligand imprinting occur in hexane but not in water?

As it was discussed above, we observed that pretreating subtilisin with an inhibitor has an effect on the conformation of the S1 pocket in hexane but not

Figure 5. Average root mean square fluctuation during the last 5 ns of the simulations in hexane (A) and water (B). The value was obtained by averaging the r.m.s. fluctuations per residue of the simulations in the corresponding solvent. The residues that correspond to the loops surrounding the S1 pocket are highlighted in gray.

in water. What are the physical determinants of these observations? It is generally accepted that enzymes are less flexible in apolar anhydrous solvents than in water and therefore they may get kinetically trapped in metastable states.^{13,19–22} To analyze the protein mobility in water and in hexane, we measured its r.m.s. fluctuations. Looking at the plots in Figure 5, we can see that the r.m.s. fluctuation values in hexane simulations are considerably lower than in water simulations. The most significant difference in average r.m.s. fluctuations corresponds to the loop formed by residues 94 to 102, which is located near the active site. These observations indicate that, in accordance with what has been observed previously, subtilisin is more flexible in water than in anhydrous hexane. In particular, the loops that surround the S1 pocket (highlighted in gray in Fig. 5) have a higher mobility in water than in hexane simulations.

In the light of these results it is reasonable to think that, due to this reduced flexibility, in hexane the S1 pocket tends to retain the conformation induced by the competitive inhibitor. This facilitates

the subsequent binding of the substrate. In water the enzyme is mobile and does not retain the configuration induced by the ligand, therefore ligand imprinting is not observed.

Concluding Remarks

This work sheds light on the molecular determinants underlying the phenomenon known as ligand imprinting. Our simulation results indicate that the inhibitor *N*-acetyl-L-tryptophan amide induces an open conformation in the active site of subtilisin. We observed that in hexane simulations the active site remained open even after the removal of the ligand. When the same assays were carried out in water, the enzyme showed a very different behavior. In this case, the structure of the S1 pocket in the ligand-treated simulations was almost indistinguishable from its structure in the ligand-untreated simulations.

Our r.m.s. fluctuation analysis supports the hypothesis that the different behavior observed in the two solvents reflects differences in protein flexibility. Enzymes in water are highly mobile and therefore rapidly “lose memory” of the ligand-induced state. This accounts for the fact that no activation is observed in reactions that take place in water when the enzyme is lyophilized in the presence of competitive inhibitors and then washed. In anhydrous apolar solvents subtilisin is rigid and therefore more likely to get “locked” in the ligand-imprinted conformation. When the reaction is carried out in an anhydrous apolar solvent, after lyophilizing the enzyme from a solution containing a competitive inhibitor, there is a higher probability that the reacting substrate will find an open S1 pocket, which would then explain the rate enhancement observed in *n*-octane.¹⁵

Materials and Methods

Protein structure selection

The structure of Subtilisin Carlsberg covalently bound to the inhibitor L-[(1*R*)-1-acetamido-2-(1-naphthyl)ethyl]boronic acid, refined at 2.65 Å (PDB code: 1AV7¹⁸) was used in our studies. This structure was selected because it contains an inhibitor that structurally resembles the ligand that we intended to dock and thus we expected the active site to be in a proper configuration to accommodate the ligand of interest. The inhibitor L-[(1*R*)-1-acetamido-2-(1-naphthyl)ethyl]boronic acid was withdrawn from the structure before the subsequent steps of this study.

Determination of protonation states

The determination of the protonation state of each titrable site in the protein was performed using a methodology developed by us, based on continuum electrostatics and Monte Carlo sampling of protona-

tion states, that has been explained in detail before.^{23,24} Only water molecules with a relative accessibility inferior or equal to 0.5 were included in the calculations of the protonation equilibrium. The electrostatic energy terms were calculated by solving the Poisson-Boltzmann equation, using the MEAD package.^{25,26} The program PETIT,²³ that implements a Monte Carlo procedure, was used to sample the protonation states at different values of pH, using the energy terms calculated by MEAD.

Docking of the inhibitor

The inhibitor *N*-acetyl-L-tryptophan amide was docked in the active site of subtilisin, using the software AutoDock, version 3.0.²⁷ This ligand was chosen because it displayed the largest rate enhancement in Russell and Klibanov's experiments.¹⁵ The inhibitor structure was built using PyMOL.²⁸ All waters were removed from the structure of subtilisin. Kollman united-atom partial charges were assigned to the protein and the ligand. Only polar hydrogens were considered. Solvation parameters and fragmental volumes were assigned using AutoDockTools (<http://AutoDock.scripps.edu/resources/adt/index.html>). This tool was also employed to determine the ligand's rotatable bonds. The program AutoGrid was used to define grid maps of 70 × 70 × 60 points, in the *x*, *y*, and *z* directions, respectively, with a 0.375 Å spacing and centered at the active site. The docking was performed using the Lamarckian genetic algorithm, with a population of 300 random individuals, a maximum number of 2.5 × 10⁶ energy evaluations, a maximum number of 27,000 generations, an elitism value of 1, a mutation rate of 0.02 and a crossover rate of 0.80. The pseudo-Solis and Wets method was used for local search, having a maximum of 300 iterations per search and a probability of performing local search on an individual in the population of 0.06; the maximum number of consecutive successes or failures before doubling or halving the local search step size was 4 and the local search was terminated when the local search step size reached a value equal or lower than 0.01. Five hundred docking runs were performed and the results were processed using cluster analysis with a root mean square (r.m.s.) deviation tolerance of 1.0 Å. The best docking solution was selected and used as a starting point for the MD simulations.

Molecular dynamics simulations

The general methodology used in the molecular dynamics simulations of proteins in nonaqueous media was developed by our group and is explained in detail elsewhere.¹³ MD simulations were performed with the GROMACS package,²⁹ version 4.0,³⁰ using the 53A6 force field.³¹ Water was modeled with the simple point charge (SPC) model³² and hexane was

treated as a flexible united atom model using the 53A6 alkane parameters.³³ Bond lengths of the solute and hexane molecules were constrained with LINCS³⁴ and SETTLE³⁵ was used for water. The simulations were performed at constant temperature and pressure. For the simulations carried out in hexane, the protein, ions and water were coupled to the same heat bath and hexane was coupled to a separate heat bath. For the aqueous simulations, the protein and water were coupled to two separate heat baths. Temperature coupling was implemented using the Berendsen thermostat³⁶ with a temperature coupling constant of 0.1 ps and a reference temperature of 300 K. The pressure control was done by applying the Berendsen algorithm with an isotropic pressure coupling, using a reference pressure of 1 atm and a relaxation time of 0.5 ps and 1.5 ps for water and hexane simulations, respectively. An isothermal compressibility of $4.5 \times 10^{-5} \text{ bar}^{-1}$ was used both for water and hexane. Nonbonded interactions were calculated using a twin-range method with short and long range cutoffs of 8 Å and 14 Å, respectively.³⁷ In water simulations, a reaction field correction for electrostatic interactions was applied,^{38,39} considering a dielectric constant of 54 for water (the dielectric constant of SPC water).⁴⁰

The necessity of using multiple replicates, in molecular dynamics simulations, has been highlighted in previous studies conducted in our group.^{13,41} It was clear in both works that a unique simulation does not capture the characteristics of the ensemble that ideally one wishes to sample. This reflects the fact that protein molecules have very complex conformational energy landscapes, with multiple minima where the system may become trapped during the simulation. To circumvent these sampling difficulties, in this study, we have used several replicates, as indicated in Figure 1 and discussed in “Overview of the simulation approach.”

Hydration conditions in hexane simulations

In their experiments, Russell and Klibanov found that the larger the water content the smaller the ligand imprinting effect. This is most likely due to the fact that there is a positive correlation between the amount of water present in an apolar organic solvent and protein flexibility.^{13,19–22} Our aim was to test the two extreme cases: anhydrous vs. aqueous conditions. Completely anhydrous conditions (0% water) are very rarely found and removing all the waters from the protein could be drastic to its stability. It has been shown that in apolar media like hexane, the water molecules are in close contact with the protein and for low water percentages, the amount of water located beyond 0.25 nm away from the enzyme surface is negligible.⁴² Thus, in our hexane simulations, we decided to keep only water mol-

ecules with a relative accessibility inferior or equal to 0.1.

Selection of counterion positions

The selection of counterion positions was done using an approach based on docking simulations, similar to the one applied before by us.¹³ A detailed description of this methodology can be found in Supporting Information, in the Section S1. Protocol for selecting counterion positions.

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