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Binding Dynamics of Two Water Molecules Constrained within the Scytalone Dehydratase Binding Pocket

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Abstract—Two water molecules reside between inhibitors and active site residues of scytalone dehydratase. A molecular dynamics study is consistent with one water molecule binding less tightly than the other. Inhibitor binding studies with site-directed mutants indicate that the hydrogen bonding network around the less mobile water molecule contributes much greater binding energy than that around the more mobile one.

Scytalone dehydratase (SD) catalyzes the conversion of two physiological substrates, scytalone and vermelone, to the corresponding naphthols in the melanin biosynthetic pathway of many fungi (Fig. 1).[1] Inhibitors of SD have been identified as potent fungicides against the rice pathogen *Magnaporthe grisea*.[2–6] The X-ray structure of SD was first solved in complex with salicylamide (**1**) (Fig. 2) to 2.9 Å and showed two crystallographic water molecules mediating contacts with the inhibitor.[7] One of the waters, which is hydrogen bonded to the salicylamide carbonyl and to the hydroxyl groups of two tyrosine residues, has a functional role in inducing enolization during the dehydration by shuttling the proton donating capabilities of the tyrosines.[8] The other water molecule, which forms hydrogen bonds with the salicylamide NH and the imidazoles of two histidine residues, has been assigned as the water of dehydration in the catalytic cycle.[7,8] We reported a higher (2.15 Å) resolution structure of SD complexed with **1**, which also shows two water molecules associated with the inhibitor.[9] Numerous high resolution X-ray crystal structures of other SD-inhibitor complexes have been solved, and with one notable exception[4] (see below), they all display two analogously positioned water molecules.[9–11] Inhibitor **1** and the associated water molecules are sequestered from solvent within the SD binding pocket. Hence, the water molecules are tightly bound creating an opportunity for improving inhibitory potency by engineering their displacement. Characterizing the nature of bound water molecules has broader implications as waters offer a structural element to the protein, especially if they are not located on the protein periphery.[12]

We recently reported the successful displacement of one of the crystallographic water molecules by a designed set of inhibitors.[4] Clearly a hydrogen bond network associated with a bound water molecule contributes an enthalpic binding energy equal to or greater than the entropic penalty of having it constrained. The value of displacing the water molecule for inhibitor design stems from the entropic energy gain by its release to bulk solvent. However, a designed displacement of the water molecule must compensate beyond this entropic energy for a portion of the enthalpic energy associated with its binding to improve binding potency. Herein we describe a molecular dynamics simulation of the two water molecules and salicylamide inhibitor **2** within the SD binding pocket to explore the relative affinities of the two water molecules for their respective microenvironments. We correlate the results of the simulation with the binding of **2** to five SD mutants. The mutated residues (shown in Fig. 2) form the hydrogen bond network around the inhibitor and, as mentioned, are important to catalysis. Through this study, we examine which water molecule might be more easily displaced by an appropriately designed inhibitor and, thereby, require a smaller return on enthalpy.

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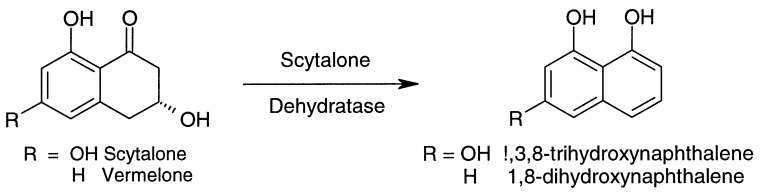

Figure 1. Reactions catalyzed by scytalone dehydratase.

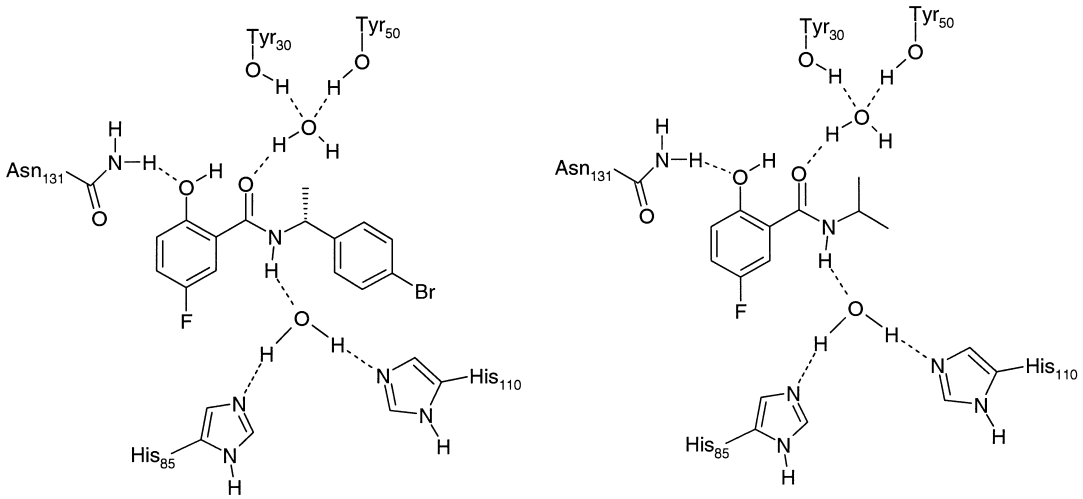

Figure 2. SD residues associated with salicylamides **1** (left) and **2** (right) and the two bound water molecules.

Results

Dynamics modeling

A molecular dynamics simulations of SD was conducted with the *N*-isopropyl salicylamide 2, the purpose of the smaller amide substituent relative to salicylamide 1 being to allow space for migration of the inhibitor and the two active site water molecules. During the 200 ps run, the protein was held rigid constraining the inhibitor and two bound water molecules by the boundaries and electrostatics of the binding pocket.[13] Table 1 shows the average energies of the stored structures from the simulation. Figure 3 shows the plot of the fluctuations of the positions of the two water molecules from their respective binding sites as a function of time. The inhibitor maintained its position within the binding pocket: the distance from the side chain carboxamide nitrogen atom of Asn131 to the salicylamide phenol oxygen atom averaged 3.4 ± 0.3 (rmsd)Å. As graphically illustrated in Figure 3A, the water molecule associated with His110 and His85 roughly maintained its original position where the distance between the NH of the salicylamide and the associated water molecule fluctuated between 2 and 4 Å. However, the water molecule associated with Tyr30 and Tyr50 migrated into the space previously occupied by the bromophenyl group of **1** as the distance to the inhibitor carbonyl gradually increased from around 2.2 Å to between 6 and 9 Å. The boundaries of

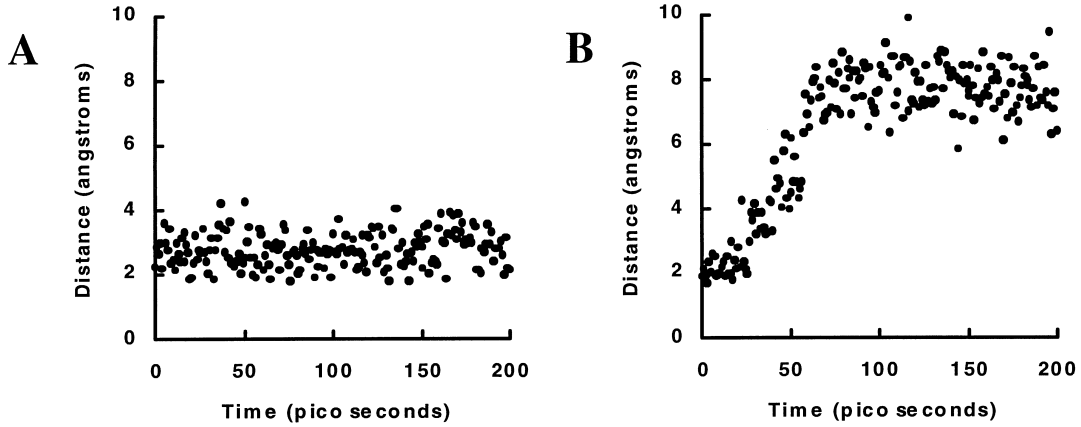

Figure 3. Dynamics simulation. (A) Distance between salicylamide NH and H_2O as a function of time. (B) Distance between salicylamide carbonyl and H_2O as a function of time.

the closed binding pocket limited the movement of the water molecule to 9 Å.

Binding of 2 to wild-type and mutant enzymes

Five site-directed mutants of SD (N131A, Y30F, Y50F, H85N and H110N) were prepared, purified, and characterized with respect to their physical and catalytic properties.[8] K_i values for salicylamide **2**[14] for wild-type and the mutant enzymes were determined using established methods[4] (Table 2). K_i values are significantly smaller than the wild type for the two tyrosine mutants; they are significantly larger for the other three mutants.

Discussion

Salicylamide **1** has a K_i of 46 pM versus SD,[9] nearly 10,000-fold more potent than **2**. The greater binding potency of **1** over **2** is due to its higher lipophilicity known to favor the partitioning from the aqueous environment[15] and to the numerous van der Waals contacts engendered by the bulky bromophenyl group with a notably hydrophobic region of the inhibitor binding pocket. Removal of the bromophenyl group for the dynamics simulation alleviates the snug association between the inhibitor and enzyme for enhancing the conformational mobility of the system and the likelihood that the inhibitor or water molecules would migrate into the newly vacated region of the inhibitor binding cavity. From the simulation, the electronic and steric constraints that keep the tyrosine associated water in position are smaller than those affiliated with the histidine associated water. The animation of the dynamics simulation shows that the tyrosine associated water molecule migrates to the hydrophobic region of the binding pocket that had been occupied by the bromophenyl group. The energies in Table 1 indicate little preference for positioning the water molecule in the region around Tyr30 and Tyr50 during the 5–32 ps period relative to the region to which the water molecule migrated during the 50–205 ps period. The dynamics simulation is fairly limited in scope since the inhibitor is purposefully changed from that used for the X-ray structure determination, since the time frame for the simulation is short, and since the enzyme is artificially constrained as a rigid body unlike what occurs in solution. However, the simulation served its purpose in this study, where it was carried out primarily to indicate which water molecule might be less tightly bound by the enzyme. That the water molecule associated with Tyr30 and Tyr50 may be less rigidly bound than that associated with His85 and His110 is supported by multiple SD-inhibitor X-ray structures that reveal greater positional variability for the tyrosine associated water molecule.[9]

Inhibitor binding with the SD mutants

Of the residues examined, Asn131 plays the largest role for inhibitor binding in accord with the hydrogen bond between the side-chain carboxamide and the salicylamide hydroxyl and with the data from the dynamics simulation. The two tyrosine mutations do not compromise binding of the inhibitor to SD. In fact, **2** binds more tightly to the Y30F and Y50F mutants than it does to wild-type SD, which may be explained, in part, by the mutation bringing a modest relief of steric strain that has an associated energy exceeding that from the loss of a hydrogen bond. The hydrogen bonding model in Figure 2 has both tyrosine hydroxyls protonating the associated water molecule, which, in turn, protonates the salicylamide carbonyl. The extent that the two protonations of the water molecule occur simultaneously is expected to be small and is consistent with the result that removal of one of the hydroxyls by mutagenesis is not deleterious to binding. In contrast, both the Y30F and Y50F mutations debilitate catalysis (by 10- and 100-fold, respectively) supporting the notion that the tyrosines each contribute hydrogens that promote the enolization of substrate scytalone.[8] It is reasonable that there might be two hydrogen bonds to the water molecule in the transition state during catalysis and only one hydrogen bond in the ground state situation of inhibitor binding. A crystal structure of a Y50F-salicylamide complex shows a clear electron density for the tyrosine associated water molecule and the conservation of the hydrogen bonds with the Y30 hydroxyl and the inhibitor carbonyl.[8] The individual hydrogen bonds from the two

Table 1. Averaged energies for the dynamics simulation[a]

Time	Potential energy (kcal/mol)	Kinetic energy (kcal/mol)	Total energy (kcal/mol)	Temperature (K)
5–32 ps	3625 ± 4	27 ± 2	3652 ± 3	290 ± 20
50–205 ps	3624 ± 3	28 ± 2	3652 ± 3	297 ± 21
5–205 ps	3624 ± 3	28 ± 2	3652 ± 3	296 ± 21

[a] ± Standard deviation.

Table 2. Binding of salicylamide **2** to wild-type and site-directed mutants of SD

Enzyme	K_i (nM)	Relative K_I's[a]	Enzyme	K_I (nM)	Relative K_I's[a]
Wild-type	450 ± 30	1	H85N	4000 ± 800	8.9
Y30F	160 ± 8	0.36	H110N	9100 ± 700	20
Y50F	85 ± 2	0.19	N131A	71,000 ± 8000	160

[a]To wild-type SD.

tyrosine hydroxyls are not necessary for inhibitor binding and, by extension, for binding of the associated water molecule. We have reported the successfully designed displacement of the tyrosine associated water molecule of SD by attaching a nitrile to quinoline and cinnoline scaffolds along with a corroborating X-ray crystal structure (1.65 Å resolution) that clearly shows the nitrile displacing the water molecule.[4] Depending on the analogue pair, the displacement of the water molecule by the nitrile afforded a 2- to 20-fold enhancement in binding to SD.

In contrast, **2** binds much more poorly to the H85N and H110N mutants than to wild-type SD pointing to a much stronger association amid the imidazoles of the two residues, the water molecule and the NH of the inhibitor. Both histidines are intimately involved in the scytalone dehydration mechanism as evidenced by the severe deterioration of the kinetic competency of the mutants.[8] In contrast to Tyr30 and Tyr50, which are thought to interact with scytalone indirectly through the water molecule, His85 and His110 are thought to associate directly with scytalone during catalysis. The salicylamide inhibitors and the NH associated water molecule are considered to mimic the transition state for catalysis, a view that is entirely consistent with the observations reported here: the histidines are more important to inhibitor (and water) binding than the tyrosines. The integrity of the 3-dimensional fold of the mutant enzymes had been established by a 1:1 stoichiometric titration with a salicylamide inhibitor,[8] so the lower binding affinity of **2** is attributed to disruption of the hydrogen bonding array with the water molecule and inhibitor. That the mutation of His85 is more deleterious to inhibitor binding than that of His110 results from there being an aspartate (Asp31) carboxylate backside to the His110 imidazole that can, thereby, become more basic and better able to accept a hydrogen from the water molecule. The greater stability of the histidine associated water molecule is in accord with the catalytic mechanism of the enzyme in that the water occupies the position of the leaving hydroxide of substrate scytalone. The enzyme is known to modulate the E1cb mechanism of the solvolytic dehydration of scytalone largely by stabilizing the leaving water molecule through the dispositions of the two imidazole groups of the histidines.[8]

In summary, the dynamics simulation indicates that the water molecule associated with the inhibitor carbonyl, Tyr30 and Tyr50 is more labile than the one associated with the inhibitors NH, His85 and His110. Corroborating the dynamics calculations, the site-directed mutations of Tyr30 and Tyr50 bind **2** slightly better than does wild-type SD, while those of His85 and His110 bind **2** considerably worse. In principle, identifying the more labile water molecule associated with an enzyme binding pocket can help prioritize inhibitor design programs. In the search for enhanced inhibitor potency, it can be expected that the lower barrier for displacement of the more labile water would make doing so by design an easier task.

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13. The molecular dynamics simulation and analysis of 2-hydroxy-*N*-(1-methylethyl)-benzenecarboxamide in the SD binding pocket were carried out with the Sybyl package of modeling programs from Tripos, Inc. (St Louis, MO). A monomer unit of the SD complex with **1** (1STD[7]) was modified by replacing the bromophenyl group with a methyl group. Valences of the protein, water molecules and inhibitor were satisfied with hydrogen atoms. Charges for all atoms were calculated using the Gasteiger–Marsili algorithm. The internal strain of a 10 Å shell around the inhibitor was relaxed using the Tripos force field with a Powell gradient to a convergence of 0.05 kcal/mol. For the molecular dynamics simulation, the modified inhibitor and the two waters were constrained by the protein that was held as an immovable aggregate. Using 1 fs time steps, the system was heated from 0 to 300 K by 50 K steps every ps and equilibrated at 300 K for a period of 200 ps. Structures were stored every ps. The Tripos force field was used for the simulation with the Verlet method of integration and the SHAKE method to hold hydrogen/heteroatom bonds at a fixed length. Non-bonded interactions were updated every 25 fs. The van der Waals energy and electrostatic terms were switched off at distances greater than 8 Å. A dielectric constant of 1.0 was used.
14. Compound **2** was prepared as described in ref 8.
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