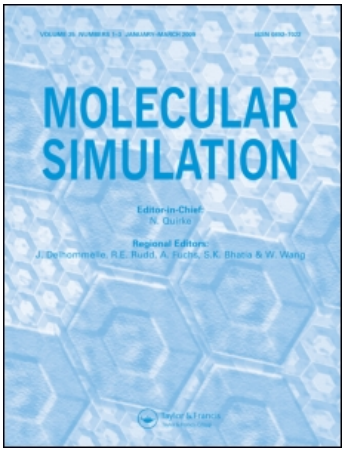

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Docking Substrates to Metalloenzymes

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DOCKING SUBSTRATES TO METALLOENZYMES

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Carbonic Anhydrase (CA) is a metalloenzyme that reversibly catalyzes the interconversion between carbon dioxide and bicarbonate anion. A class of sulfa drugs, sulfonamides, are known to inhibit CA. One approach to identifying important binding and specificity interactions between sulfonamides and CA is to analyze the results from docking studies. Previous docking studies have mainly focused on the encounters of substrates with non-metalloenzymes. Here we report the application of MOE-Dock to the CA II – sulfonamide system. After developing a standard docking protocol for the CA II – sulfonamide system we then used the protocol to determine other CA II – sulfonamide complexes.

Keywords: Docking substrates; metalloenzymes

INTRODUCTION

Docking methods have developed to a point where one can determine the position of a substrate bound into the active site of an enzyme. The main goal of automated docking procedures is to determine the "best" substrate–receptor complex. Figure 1 illustrates the docking of a substrate to a receptor. In this example, not only is there a shape complementarity, but also charge interaction specificity. For a docking method to be useful it must be accurate and have low computational cost.

*Corresponding author.

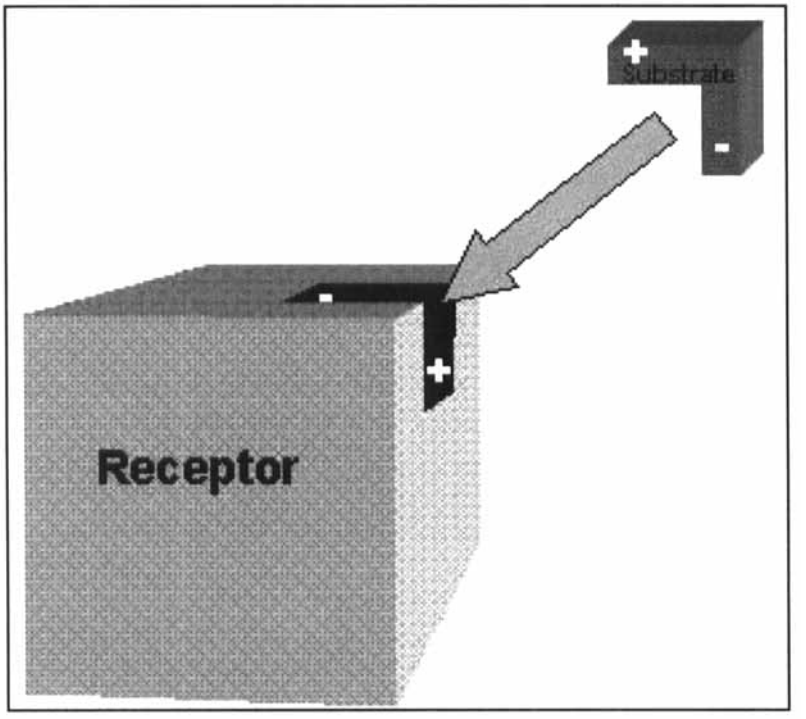


FIGURE 1 Example of substrate–receptor interaction. The substrate will fit into the receptor, based on geometry, in two different conformations. However, taking into consideration the charges on the substrate and the charges in the active site there is only one correct docking conformation.

The Binding of Substrates to Metalloenzymes

Many docking studies thus far have focused on non-metalloenzymes. Metalloenzymes are an important class of enzymes. They are used to regulate CO_2 levels in the body, catalyze the conversion of fatty acids to amides, dioxygen and electron transport and structural roles. In this paper we report our efforts on the docking of sulfonamides to Carbonic Anhydrase II (CA II), a metalloenzyme. This system was chosen because of the available structural and binding data. We started with a known CA: sulfonamide complex and using MOE-Dock we were able to dock the substrate into the active site successfully reproducing the X-ray structure.

CARBONIC ANHYDRASE

CA's general shape is ellipsoidal dominated by an internal structure of a twisted pleated sheet containing 10 β sheets. The β sheets travel through

the center of the enzyme in an antiparallel configuration. The active site is a conical shaped cleft 15 Å in depth with an entrance approximately 15 Å wide. At the bottom of the active site near the apex of the cone is a zinc metal ion with a +2 charge. The position of the zinc ion is approximately the center of the enzyme. The zinc ion is connected to the imidazole group of three histidines, His-94, His-96 and His-119, forming the base of a tetrahedron.

Function in Mammals

Carbonic Anhydrase is a hydrolytic enzyme, which catalyzes the addition and removal of the elements of water in a substrate molecule. Roughton and Booth determined the CA catalytic activity depends on pH and the ionic composition of the reaction medium. Under normal experimental conditions, an ionizing group having a pK_a near 7 controlled activity. This ion has the ability to lower the pK_a of coordinated oxygen-containing ligands while avoiding the potential for undesired electron-transfer chemistry, since divalent zinc does not have any readily accessible redox states. The zinc metalloenzymes that make-up the CA family can perform the reversible hydration of carbon dioxide in a two-step reaction to yield bicarbonate and a proton. This is an essential process for many physiological anion exchange processes [1].

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+ \quad (1)$$

Carbonic Anhydrase II (CA II) is an enzyme that exhibits reaction kinetics approaching the limit of diffusion control with a $k_{cat}/K_M = 10^8\,M^{-1}s^{-1}$ [2].

Different Isozymes

Seven different isozymes of the mammalian form of CA have been identified with distinct activities, tissue specificity and physiological roles. High concentrations of CA I and CA II are expressed in red blood cells. CA III is present in the cytoplasm of muscle tissue cells. The only known mammalian membrane bound isozyme is CA IV, which is bound to the membrane in the lung, kidney, brain and eye tissues. CA V is expressed in the mitochondria, while isozymes CA VI and VII are detected in saliva and salivary glands.

Interest in CA II and CA IV

CA II and CA IV are of interest due to their implications in glaucoma therapy, large quantity of these two isozymes are found in the eyes of

mammals. The active sites of both are similar, a 15 Å deep conical cleft with the zinc metal-ion (+2) positioned at the bottom [3]. CA II is the most active of the cytosolic isozymes for the catalysis of CO_2 hydration [4].

The major difference between CA II and CA IV is the structural difference in the wall of the active site in the region of the 130's residue segments approximately 6 to 8 Å from the zinc. In human CA II this region is a three-turn α-helix, while in human CA IV the region is loop which is believed to hold this isozyme to the membrane of the cell. The overall structure of CA II is not significantly changed when a substrate or inhibitor is bound to the active site. The noticed changes between the native and the bound structures occur in the side-chains of surface residues [5].

Immunochemical studies have determined that CA II is the most abundant isozyme of CA distributed in the eye with high concentrations of CA II in epithelial cells of the ciliary process, in Muller cells of the retina and in a subset of cone photoreceptor cells [6]. CA II and CA IV, another highly active isozyme, catalyze bicarbonate (CO_2) in the aqueous humor that is related to elevated intraocular pressure.

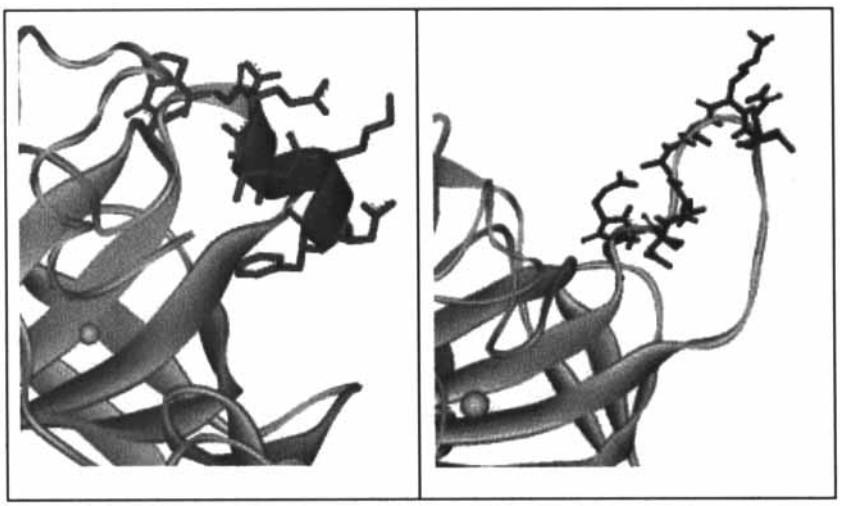


FIGURE 2 These structures are CA II and CA IV respectively focusing on the 130's residue region. The macromolecule on the left is human CA II, the red tube structures are residues 130 to 139 forming a three-turn α-helix. The structure on the right is human CA IV where the same residues are blue tube structures forming a loop thought to attach the macromolecule the membrane of the cell. (See Color Plate V).

Substrate Affinity

It has been determined that ionized sulfonamide nitrogen, 1 −, displaces a zinc-bound hydroxide to form a stable inhibitor−enzyme complex with nanomolar affinity. The similarity of CA II and CA IV's susceptibility to aryl sulfonamide inhibition, an effective method to regulation of intraocular pressure (IOP), make aryl sulfonamide of interest in glaucoma therapy. Mann and Keilin [7] found that aromatic sulfonamides having an unsubstituted $R-SO_2NH_2$ compounds are strong and specific inhibitors of CA. The sulfonamide group interacts with the zinc ion, the OH group and the amide NH group of the amino acid Thr-199. The pH dependence of sulfonamide binding is in accord with equilibrium between the ionized inhibitor and the low pH form of the enzyme, or the equivalent equilibrium between the un-ionized inhibitor and the high pH form of the enzyme [8]. The equilibrium rate of sulfonamides with CA is slow causing sulfonamides to perform as pseudo-irreversible inhibitors of CO_2 hydration and HCO_3^- dehydration.

Glaucoma and Therapy Implications

Glaucoma is a specific pattern of optic nerve damage and visual field loss caused by a number of different eye diseases. Most, but not all, of these diseases are characterized by elevated intraocular pressure (IOP), which is not the disease itself, but the most important risk factor for the development of glaucoma. Glaucoma usually occurs without apparent symptoms, therefore, a person with glaucoma is usually unaware of its onset until serious loss of vision has occurred. It is approximated that half of those afflicted are not aware they have glaucoma and the damage from glaucoma cannot be reversed. There is no way to prevent glaucoma, only recommended treatment to minimize the risk or the effects of this disease. Individuals at risk of developing glaucoma are those with a family history, myopia (nearsightedness), diabetes, high blood pressure, a previous eye injury, long exposure to cortisone and African descent.

Sulfonamide inhibition of CA enzymes is considered one of the leading treatments for glaucoma, yet the delivery route of the inhibitor to the eye is of concern. Oral consumption of sulfonamide compounds while relieving IOP produce unwanted side effects such as electrolyte imbalances, gastrointestinal disturbances, development of renal stones, general malaise and other diseases of the blood. It has been determined that more than 99.9% [9] of the CA enzyme in the eye must be inhibited to reduce IOP. The

complications associated with oral CA inhibitors has lead to the development of topical sulfonamide solutions for direct application to the eye. While the topical method of treatment is better overall, the eye is able to clean itself quickly, washing away the inhibitor solution. If the remaining inhibitor penetrates slowly into the eye, then a sufficient concentration of the inhibitor cannot accumulate to reduce the production of the aqueous causing IOP. Penetration through the lipophilic cornea by sulfonamides is poor because of the structure of the inhibitor; the sulfonamide group is dissociated at physiologic pH and thus polar and hydrophilic.

Docking Programs

Docking programs belong to two classes, 'direct' and 'unbiased'. Direct has the advantage of speed, yet the disadvantage of making assumptions about the potential energy landscape. The unbiased method makes few assumptions about the potential energy landscape thus finding final docked solutions that the direct method might have missed, yet this is at the expense of computation time. There are several docking programs available. We have chosen to use MOE-Dock by Chemical Computing Group Inc., which is based on the program AutoDock® 2.4 by Art Olsen at Scripps Research Institute. Both of these programs fall under the classification of an unbiased method.

MOE-Dock

MOE-Dock utilizes a MC simulated annealing process similar to that used in AutoDock® 2.4 for docking a substrate into the active site.

A three-dimensional grid is constructed surrounding the coordinates for the protein target. A probe atom visits each grid point, the interaction energy is calculated and stored. The completed grid of energies provides a look-up table for the rapid evaluation of interaction energies. Separate tables are calculated for each type of atom in the ligand, including dispersion–repulsion and hydrogen bonding energies. A separate electrostatic potential grid is calculated, using a probe carrying a single positive charge. The electrostatic interaction energy for each atom in the ligand may then be obtained during the simulation as the product of the local value taken from this grid and the partial charge on the atom. MOE-Dock allows the substrate to search six spatial degrees of freedom, rotational and translation and any number of torsional degrees of freedom perform the actual docking simulation. However MOE does not allow conformations where

an atom(s) of the substrate are protruding from the grid box, an energy of infinity is assigned. Whereas in AutoDock® the location of the substrate is manipulated so it remains in the grid box; this does not prohibit atoms from leaving the box in AutoDock® but instead the atoms outside of the box are assigned interaction energy of zero. A random perturbation to each is applied at each step and the interaction energy evaluated for the new location and conformation. The new state is either accepted or rejected probabilistically based on the annealing temperature of the system [10]. This probability depends on the energy and temperature of the cycle. If a cycle is at a high temperature, many conformation states will be accepted. If a cycle is at a low temperature, many of the probabilistic states will not be accepted. Through this process the ligand will usually find the minimum energy of the deepest energy well on the potential energy surface. Starting separate simulated docking attempts at random starting locations will usually lead to consistently similar binding orientations.

METHODS

Parameters

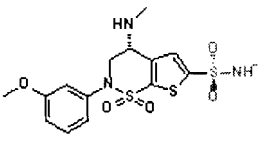
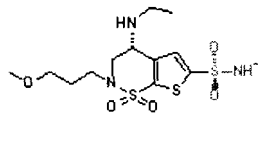
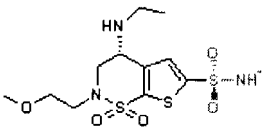
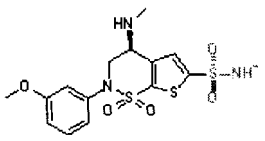
The edited PDB crystal structure (water molecules removed) of CA II was imported into MOE and hydrogen atoms were added. The charges of the individual atoms of the enzyme were then calculated using the Kollman 89 method and the zinc metal ion was set to a +2 charge and ionization of 2+. The partial charges of the inhibitor atoms are Electro-Static Potential (ESP) calculated through AM1 geometry optimization in Spastan. The inhibitors of interest are in Table I. A comparison of the AM1 and Hartree-Fock 6−31G* partial charges for AMS was performed.

The charges determined by AM1 are comparable to those of Hartree-Fock as seen in Chart 1. AM1 performs well in comparison to Hartree-Fock for the partial charges of both hydrogen and carbon atoms. The AM1 partial charges of sulfur, oxygen and nitrogen atoms are not similar to Hartree-Fock values, the AM1 partial charges do scale correctly. The van der Waals radius for hydrogen atoms bonded to carbons was set to 1.20 Å to also correspond with known experimental values.

MOE Dock Settings

In the Dock portion of MOE a grid-box of $70 \times 70 \times 70$ points with a grid spacing of 0.375 Å was centered at −6.8680, 2.6221, 15.9747, 4.258 Å

TABLE I Inhibitors of CA II

Name	*Structure*	K_d (*nM*)	*Label*
(*R*)-3,4-dihydro-2-(3-methoxyphenyl)-4-methyl-amino-2H-thienol[3,2-e]-1,2-thiazine-6-sulfonamide-1,1-dioxide		0.10 ± 0.01	IN3R
(*R*)-4-ethylamino-3,4-dihydro-2-(3-methoxy)-propyl-2H-thieno[3,2-e]-1,2-thiazine-6-sulfonamide-1,1-dioxide		0.13 ± 0.03	AZP
(*R*)-4-ethylamino-3,4-dihydro-2-(2-methoxy)-propyl-2H-thieno[3,2-e]-1,2-thiazine-6-sulfonamide-1,1-dioxide		0.32 ± 0.05	IN2R
(*S*)-3,4-dihydro-2-(3-methoxyphenyl)-4-methyl-amino-2H-thienol[3,2-e]-1,2-thiazine-6-sulfonamide-1,1-dioxide		1.70 (single)	IN3S

above the zinc atom. In each trial the number of Runs and Cycles was set to 25 while the Accept/Reject limit was set to 30,000 iterations. One trial was performed for each inhibitor.

Choosing the Predicted Docked Structure from MOE

Each trial was examined for predicted docked structures with the lowest energies. The database of predicted docked structures was examined for configurations with the lowest energies of the trial. The configurations with the lowest energy yet wrong orientation of the substrate in the receptor site, *i.e.*, sulfonamide group of the inhibitor was not interacting with the zinc metal ion, were discarded. Knowledge that the sulfonamide group of the inhibitor interacts with the zinc atom of CA is vital and is known only through numerous experimental data primarily X-ray structures. The remaining structures were considered for examination using the Energy Minimization Methods described below and University of Houston

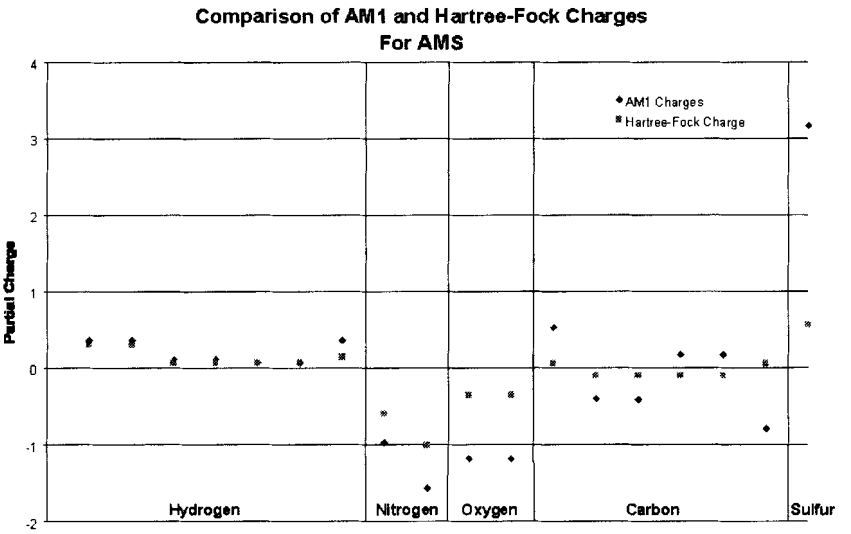


CHART 1 Comparison of AM1 and Hartree-Fock charge for AMS.

Brownian Dynamics (UHBD). Structures of additional interest were those with high-energy values yet similar orientation to structures with low energy.

Energy Minimization After Docking

After good predicted docked structures were found, minimization was performed to refine the orientation of the substrate in the receptor site. Energy minimization is used to find the *global energy minimum* of the substrate–receptor interaction. The global energy minimum is considered the lowest energy of the energy potential surface, thus the most stable conformation of the system. For large systems, such as enzymes, the global energy minimum can be difficult to find due to the possibility of many *local energy minimums* on the energy potential surface. These local energy minima are the result of shallow clefts on the surface of the enzyme where the substrate will bind favorably in simulated docking trials. The energy minimization function in MOE uses a sequential combination of three different minimization techniques in each step of the minimization process. The first technique is Steepest Descent which moves in the direction parallel to the net force, similar to walking down a hill in a path of least resistance. The next technique is the Conjugate Gradient where the gradients at each point are orthogonal but the direction is conjugate, whereas in the Steepest

Descent method both the gradients and the direction of successive steps are orthogonal. The third minimization technique is a Truncated Newton-Raphson technique that eliminates the off-diagonal elements for large distance interactions between atoms. The Newton-Raphson method finds the minimum in one step from any point on the energy surface. Two methodologies were used for the minimization of the system. Minimization Method 1 (MM1) fixes all atoms of the receptor, the substrate is allowed to relax into the active site. In Minimization Method 2 (MM2) the atoms of the active site were allowed to move while the rest of the receptor including the backbone structure of the active site was fixed. Table II contains the amino acid and residue number of CA II residue side-chains not fixed.

UHBD

The use of the UHBD program was for it's calculation of binding energies between the substrate–receptor. While the Interaction Energy values from MOE are useful, they were not considered correct. Validation of UHBD consisted of determining the correct type of partial charges for the atoms of the receptor. Electrostatic Energy Minimization (EEM) charges where deemed correct. EEM computes the partial charges according to a predetermined partial charge model for all atoms in the system, the receptor. The EEM charge model consists of an energy function as a function of charge for each atom in the system, along with Coulomb electrostatic energy terms. The partial charge depends upon molecular conformation. At the completion of the assignment of partial charges the zinc metal ion charge was set to a value of +2 to reflect the value used in the simulated docking. These procedures were validated against both experimental and theoretical values [11]. Using the solved X-ray structure of CA II with bound AMS (with 6.31 G* charges) an Absolute Free Energy of Binding (AFEB) was calculated, −8.06 kcal/mol. This value is in excellent

TABLE II Residue numbers and type of amino acid lining the active site of CA II

Residue number	*Amino acid*	*Residue number*	*Amino acid*
7	Tyr	131	Phe
64	His	141	Leu
65	Ala	143	Val
67	Asn	198	Leu
91	Ile	200	Thr
121	Val	207	Val

agreement with the experimental value of Maren and theoretical value of Madura *et al.*; −8.3 and −8.1 kcal/mol respectively. The calculated value of −8.06 kcal/mol is the average of seven different hand manipulated orientations of the AMS molecule. An average value of −18.70 kcal/mol was calculated by translating the AMS molecule ±0.1 Å in the *X*, *Y*, *Z* directions from that of the X-ray solved structure. To eliminate the possibility of the substrate being located on a grid point during a UHBD calculation the substrate was translated ±0.1 Å in the *X*, *Y*, *Z* directions. Using AM1 ESP partial charges on the AMS molecule was also translated ±0.1 Å in the *X*, *Y*, *Z* directions from the X-ray solved structure and an average value of −7.51 kcal/mol was calculated. At the completion of the MM2 process the EEM partial charges of the atoms for the receptor were calculated to reflect the change in active site side-chain conformation. These values and the UHBD binding energy value for the original location of the substrate were averaged. UHBD calculations were conducted for the original predicted structure, the MM1 structure and the MM2 structure. Values are listed in Table III.

RMSD Calculations and Values

The Root Mean Squared Deviation (RMSD) value shows the relationship of the change in UHBD binding energies with respect to the overall

TABLE III Summary of data

	IN3R #3	*AZP* #2	*IN2R* #18	*IN3S* #14
MOE Dock Energy (kcal/mol)	−27.013	−24.720	39.632	42.874
UHBD AFEB (kcal/mol)	−17.904 ± 1.480	−20.186 ± 2.302	−2.391 ± 0.489	1.156 ± 1.473
MOE Interaction Energy MM1 (kcal/mol)	−287.770	−241.597	−157.905	−104.718
UHBD AFEB MM1 (kcal/mol)	−29.761 ± 2.460	−22.032 ± 3.562	−2.696 ± 1.067	0.913 ± 0.692
RMSD Original Predicted Structure (Å)	1.415	0.601	1.055	0.476
MOE Interaction Energy MM2 (kcal/mol)	−299.912	−270.005	−208.123	−174.747
UHBD AFEB MM2 (kcal/mol)	−30.222 ± 2.564	−21.368 ± 3.424	−0.664 ± 0.326	1.470 ± 0.917
RMSD Original Predicted Structure (Å)	1.331	0.650	0.926	0.708
RMSD MM1 Structure (Å)	0.160	0.509	0.894	0.740
K_d (nM)	0.10 ± 0.01	0.13 ± 0.03	0.32 ± 0.05	1.70 (single)

movement of the substrate induced by the minimization methods. RMSD calculations are performed using the equation:

$$\text{RMSD} = \sqrt{\frac{\sum(R_i - R_i^{\text{ref}})^2}{n}} \tag{2}$$

where R_i are the x, y, z coordinates of the individual atoms of the substrate of interest, R_i^{ref} are the x, y, z coordinates of the individual atoms of the reference substrate, and n is the number of atoms in the structure. As seen in Table III a RMSD of approximately 1.3 Å can cause the energy to change by as much as 18 kcal/mol.

RESULTS AND DISCUSSION

Predicted Docked Structures from MOE

The structures with lowest docking energies were chosen for further evaluation. There were several docked structures with low binding energies yet were considered incorrect docked structures. An incorrect docked structure was when the sulfonamide group was not interacting with the active site, one of the side chains of the substrate interacts with the active site or when the substrate is docked outside of the active site. For most cases the structure with the lowest docking energy was the best-docked structure.

Energy Minimization and RMSD Values

The energy minimization of the substrates into the receptor proved useful because of its ability to reduce the UHBD binding energy. In the cases of MM1 and MM2 the reduction of UHBD binding energies averaged approximately 3.5 and 4.1 kcal/mol respectively. This reduction in UHBD binding energies can be directly related to the minimization of substrate and the active site. When starting a minimization with different conformers in the gas phase one expects to achieve the same minimized structure. The minimizations in this study are not in a gas phase, rather the minimizations are occurring in an enzyme. The concept that through energy minimization of different predicted docked structures the same orientation will be the outcome has not worked. This can be attributed to different areas of the active site attracting or repelling the substrate. Depending on the original location of the substrate is located the interactions might

be stronger or weaker than other substrate–receptor interactions of similarly predicted docked structures. RMSD values help to illustrate the change in UHBD binding energy as compared to change in location of the substrate.

UHBD

The UHBD binding energy values were used instead of the MOE Potential Energy Interaction values because UHBD AFEB reflect experimental values and Potential Energy Interaction values are not truly descriptive of substrate–receptor binding. Using the same parameters and methods noted before for the validation of UHBD the best predicted docked structures were minimized, using MM1 and MM2, and UHBD calculations were performed. In Table III the all the energy values and RMSD values for the best-predicted dock structure of the individual inhibitors are present.

The UHBD AFEB values for both the MM1 and MM2 correspond to the experimental K_d values of Boriack-Sjodin *et al.* Also notice the MOE Interaction Energy after the minimization methods also correlates to the experimental values, yet these values are not as reliable as the UHBD values.

SUMMARY

Using a standard protocol it is possible to correctly predict not only the correct orientation of substrates to a specific metalloenzyme, but also predict the affinity of a substrate from a group of substrate to that specific metalloenzyme. While it is not possible to directly predict the experimental K_d value from the Absolute Binding Free Energy it is possible to rate different substrates for a specific enzyme.

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