Biomolecular Design and Receptor-Ligand Interaction of a Potential Industrial Biocatalsyt: A Thermostable Thermolysin-Phosphoethanolamine-Ca^{2+} Protein Complex

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Abstract: Protein structures are prone to modification based on the fundamental rules of design and function. Calculations of free binding energies (ΔG) of chemical molecules (effectors) that bind to proteins are important in molecular signaling processes and catalytic mechanisms of certain key enzymes. These calculations can be obtained via in silico and theoretical approaches. A series of 48 pockets were identified in thermolysin (KEI) and the four biggest pockets were selected for their suitable sites for modification. Application of molecular docking on phosphoethanolamine (PSE) and 1,10-phenanthroline (PHN) that act as intermediate ligands in the designated protein complex showed favorable final docked energy at different pockets (-8.49 to -4.80 kcal/mol). Analysis on docking of a divalent metal ion (Ca^{2+}) to ligand (PSE) produced a final docked energy of -4.15 kcal/mol within acceptable distance (1.5 Å ≤ M ≤ 3.0 Å). It was found that the thermolysin-phosphoetanolamine-Ca^{2+} represented the putative protein complex of semisynthetic metalloprotease. Combinatorial modeling methods were applied in order to determine the best metalloenzyme complex. The identification of the potential protein pocket was conducted using CASTp. Selected ligands and metal ions were docked into each pocket using AutoDock 3.05. Analyses on their docking energy, non-covalent interaction as well as their geometry were conducted in order to determine the best metalloenzyme complex. This complex displayed the lowest docking energy with the additional Ca^{2+} suitably docked. It was hypothesized that metal ions can add new functionality to proteins and catalyze some of the challenging biological reactions, particularly in the pharmaceutical and fine chemicals industries.

Keywords: Thermolysin, Metalloenzyme, Molecular docking, Biocatalyst.

1. INTRODUCTION

Enzymes are mainly biomolecular polymers that are able to catalyze chemical reactions. For many years, researchers have utilized the diverse chemical reactions driven by enzymes in biotechnological industries. One such industrial application known as bioprocessing, aims to exploit enzymes in many industrial processes. The development of such enzymatic tools, however, requires a detailed structural and chemical understanding of the enzyme. Enzyme engineering is an invaluable tool for elucidating biocatalytic mechanisms as well as producing enzymes for industrial purposes. Approaches developed for *in vivo* chemical modification and *in silico* modification promises to increase the scope to alter existing proteins for better stability and functionality [1]. Through *in silico* molecular design, three-dimensional conformations and enzymatic mechanisms can be clearly predicted and improved, which lead to a better understanding of the fundamentals of protein chemistry.

The electrostatic environment at the active site of an enzyme is one of the major factors that guide the substrate to the binding site in the correct position. Metal ions can contribute positively in this process, often binding groups in a stereochemically rigid manner, thereby helping to control and enhance the activity of the enzyme [5]. The goal was to tailor and analyze the dependence of the binding sites of thermolysin on a bound ligand and metal ion. Included in this report is a recently concluded systematic structural study on molecular docking between thermolysin and Ca^{2+} divalent metal ion which attached to the intermediate phosphoethanolamine (PSE) ligand. The properties of a semisynthetic metalloenzyme that illustrates the importance of ligand diversity and metal ion binding to protein were also recognized.

2. MATERIALS AND METHODS

AutoDock (Version 3.05) is a widely used program that was employed to generate an ensemble of docked

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conformations for each ligand molecule to protein [13]. Sausa *et al.* reported that AutoDock had become the most frequent docking software since 2001 [15]. Generally, this molecular docking program can be divided into three main programs: AutoGrid, AutoTor and AutoDock. In order to run AutoDock, grid maps have to be calculated using AutoGrid. For consistency, all receptor-ligand interactions were prepared using the same parameters; (i) number of grid points were set to 60 Å × 60 Å × 60 Å in x, y and z axis, (ii) spacing between grid points was set to the default value of 0.375 Å, and (iii) a grid center was chosen slightly off the center axis of the crystal structure coordinates of themolysin specific pocket. In this study, the Lamarckian Generic Algorithm (LGA) was selected to identify the binding conformations of the complex [13]. The step size was set to 0.2 Å for translation and 5° for orientation and torsion. The other important parameters for LGA calculation were reasonably set as follows; (i) an initial population of random individuals with a size of 50; (ii) a maximum number of 1.5×10^6 energy evaluations; (iii) a maximum number of generations of 27,000; (iv) an elitism value of 1 for surviving the step into the next generation; (v) a mutation rate of 0.02, which was the probability that a gene would undergo a random change; and (vi) a crossover rate of 0.80, which was the probability proportional selection. The pseudo-Solis and Wets local search method was applied by having a maximum of 300 iterations per local search; the probability of performing local search on an individual in the population was 0.06; the maximum number of successes or failures was 4, in both cases; and the termination criterion for the local search, was 0.01.

2.1. Preparation of Target Molecule, Ligands and Metal Ions

The coordinate of thermolysin-substrate free structure coded as 1KEI was downloaded from the Brookhaven Protein Data Bank (PDB) [16]. The thermolysin structure was allocated for their polar hydrogen atoms and each atom was assigned using Kollman to united atom charges. Two chemical ligands, phosphoethanolamine (PSE) and phenanthroline (PHN) selected for screening were also obtained from PDB. Their flexibility and torsion were defined through AutoTors. Two different divalent metal ions from alkaline earth metals (Mg^{2+} & Ca^{2+}) and transition groups (Fe^{2+} & Zn^{2+}) were selected for docking to the protein-ligand complex. Protein pockets were identified using Computational Atlas Topography of Protein (CASTp) [11], an online resource for locating, delineating and measuring concave surface regions on three-dimensional structures of proteins.

3. RESULTS AND DISCUSSION

Interactions between a protein, called a receptor, and a small molecule, called a ligand, usually occurs in depressed regions, called pockets, on the surface of the receptor. Geometric approaches to recognize pockets on a protein usually involve the definition of surfaces on a protein. The three-dimensional structure of protein provides the necessary shape and physico-chemical texture to facilitate these interactions [14]. Structural information of protein pockets allows for detailed study of the relationship between protein structure and function, as these pockets accommodate ligands, prosthetic groups or functional water molecules through conformational flexibility [12]. Moreover, identification and size characterization of surface binding sites or protein pockets are the initial steps in protein structure-based design [2].

A series of 48 pockets were identified in thermolysin, where the pockets are widely distributed on the protein surface. Four of the largest pockets (No. 45, 46, 47 and 48) in thermolysin were obtained from CASTp (Figure **1**). The pockets were determined by locating and measuring protein topology in solvent accessible surface (SA, Richard's surface) and molecular surface (MS, Connally's surface) [11].

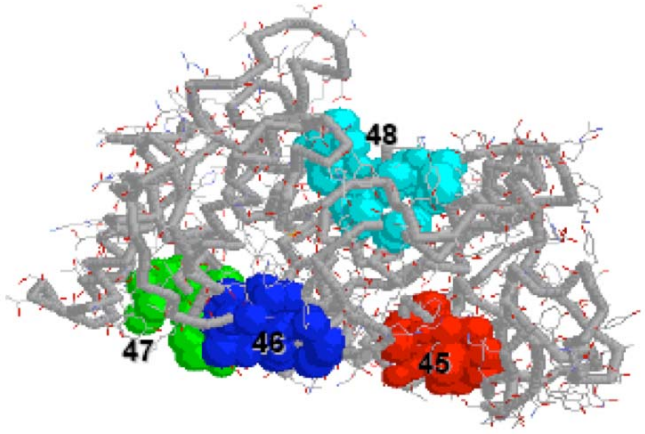


Figure 1: Visualization of thermolysin (1KEI from PDB) with four main pockets as determined by CASTp. The possible binding sites are shown as pocket **48** (Cyan), Pocket **47** (Green), pocket **46** (Blue) and pocket **45** (Red).

3.1. Protein-Ligand Docking

It is important to treat the ligand as a flexible molecule mimicking the structure that naturally associates with high intramolecular strain, which is

largely offset by intermolecular interaction enabled by its fit [3]. The ligand flexibility and constraint was determined using AutoTor. This utility interactively queries the user about rigid position of the molecule (the "root") and the rotatable torsions (the "branches" and "torsion"). Once the ligand's flexibility has been identified, the ligand can be docked to the protein in flexible form in order to discover the best outfit with the lowest docking energy. Davies and Distefano reported that for a successful design of semisynthetic metalloenzymes with novel properties such as the ALBP-Phen-Cu(II) complex, additional metal ions must be isolated from the protein with minimum interaction with the protein [5]. By considering some possible non-covalent interactions, phosphoethanolamine (PSE) and phenanthroline (PHN) were selected as the intermediate ligands. They function to hold the metal ion at one end and the protein at the other end via non-covalent interactions involving oxygen and nitrogen atoms available in the ligand.

With some minor exceptions of disulfide bonds, it is the non-covalent interactions that are responsible for the three-dimensional structure of proteins. The two most vital non-covalent interactions are hydrogen bonding and hydrophobic interaction. Moreover, Kahraman *et al.* (2007) discovered that the hydrophobicity of the binding pocket seems to correlate with the properties of the ligand bound to the protein [9]. Table **1** shows the lowest E_{docked} (kcal/mol) for each ligand docked to the four main pockets in thermolysin. The conformation of KEI-PSE48 complex at the lowest docking energy (-8.49 kcal/mol) formed six hydrogen-bond interactions and five hydrophobic contacts with nearby residues, particulary at the catalytic sites of His146 and Glu166 which may able to inhibit the catalytic activity. Therefore, PSE ligand may have acted as a competitive inhibitor to the substrate at pocket 48 and not suitable for further modification. Interestingly, the activity of PSE at pocket 45 (-6.71 kcal/mol as shown in Table **1**) involved two hydrogen-bonds and 11 hydrophobic interactions with the neighboring residues (Table **2**). The distance between pocket 45 and the active site could make it a good location for further modifications with metal ion.

3.2. Metal Ions Docking Analysis

Two different types of divalent metal ions from alkaline earth metals (Mg^{2+} & Ca^{2+}) and transition groups (Fe^{2+} & Zn^{2+}) were docked to the ligand site of the KEI-PSE45 complex. To verify the final conformation of the new semisynthetic metalloenzyme, an array of procedures was introduced; 1) Final docking energy – only spontaneously and thermodynamically stable conformations with negative docking energy were selected for further modification, 2) Allowed metal ions distance – docking results of each metal ion were

Table 1: Final E_{docked} (kcal/mol) of Two Intermediate Ligands at Four Largest Pockets in Thermolysin as Calculated by AutoDock 3.05

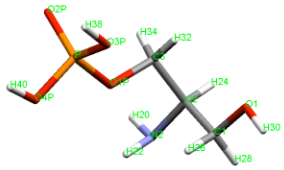
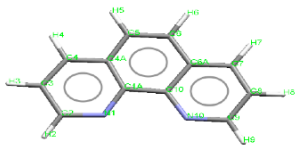
Protein-Ligand Complexes	Final E_{docked} Energy (kcal/mol)			
	Pocket 48	Pocket 47	Pocket 46	Pocket 45
 KEI – PSE (phosphoethanolamine)	-8.49	-6.74	-5.80	-6.71
 KEI – PHN (phenanthroline)	-7.06	-6.60	-5.70	-6.06

Table 2: Visualization of Docking and the Non-Covalent Interactions Within the Complex of KEI-PSE45 Using Viewer Lite and Ligplot 4.0

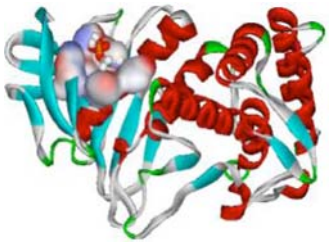
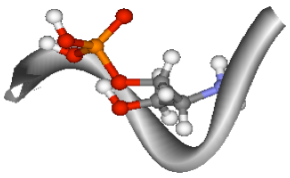
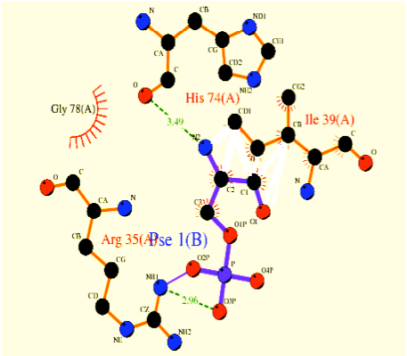
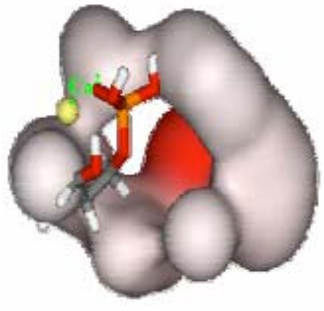
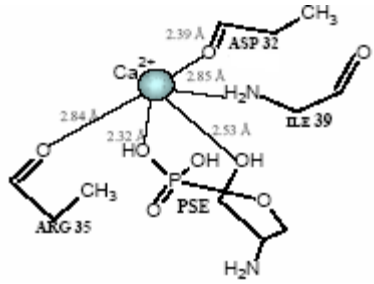
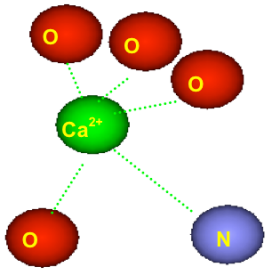
Docking Orientation of PSE Ligand onto Pocket 45	3-Dimensional Position for Protein-Ligand Complexes	Non-Covalent Interactions for Protein-Ligand Complexes
	 Final E_{docked} = -6.71 kcal/mol H-bond interactions = 2 Hydrophobic interactions = 11	

Table 3: Visualization of Docking and Geometrical Analysis of KEI-PSE45-Ca

Visualization of the New-Complex (KEI-PSE45-Ca^{2+})	Acceptable Interaction Distance of Ca^{2+} with Nearby Residues in KEI-PSE45 Complex	Ca^{2+} Geometry
		
* Pocket 45 only	Final E_{docked} = -4.13 kcal/mol	No. of contacts = 5 Type of geometry = Distorted trigonal bipyramidal

studied for their 3-dimentional conformations using InsightII. An average distance of metal ions proposed by Castagnetto *et al.* (2001), 1.5Å ≤ **M** ≤ 3.0Å was set as the criterion [4]. Only the metal ions within the cutoff distance from the ligands were selected for further investigation, 3) Types and angles of metal ions complex – the coordinates for each metal ion were analyzed for their type and specific geometry. The conformations were then compared with other crystallized metalloenzymes in the Metalloenzyme Data Base (MDB) for their type and geometry.

The Ca^{2+} ion was found to have the lowest E_{docked} of -4.13 kcal/mol. The final Ca^{2+} conformation was noticed to interact with the PSE intermediate ligand and nearby residues within the acceptable distance of interactions. As shown in Table **3**, the KEI-PSE-Ca^{2+} complex formed a distorted trigonal bipyramidal geometry by coordinating to five neighboring atoms. The geometry is similar to the reported trigonal bipyramidal geometry in rat annexin V protein complex coded as 1a8a [17].

4. CONCLUSIONS

An important branch of novel protein design is performed through engineering and designing of new metal-binding sites in native proteins. Metal ions can add new functionality to proteins and help catalyze some of the most difficult biological reactions. By employing *in silico* molecular docking, screening of putative ligands and metal ions for possible interactions may enhance the discovery of novel semisynthetic enzymes and lead to new protein functions. The framework which was introduced for the experiment may be a competent method for screening potential metal ions in this *in vivo* route.

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ABBREVIATIONS AND NOTATIONS

CASTp = Computed Atlas of Surface Topography of Protein

MDB = Metalloenzyme Database

LGA = Lamarckian Generic Algorithm

PSE = Phosphoethanol amine

LigPlot = Schematic diagrams of protein-ligand interactions

PDB = Protein Database

1KEI = Thermolysin

PHN = Phenanthroline

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