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Urease Inhibition

Muhammad Raza Shah[1] and Zahid Hussain Soomro[2]
[1]*International Center for Chemical and Biological Sciences, H.E.J. Research Institute of Chemistry, University of Karachi*
[2]*Institute of Materials Science and Research Dawood College of Engineering and Technology Karachi*
Pakistan

1. Introduction

The design, synthesis, characterization and exploring the broad spectrum of potency of biologically active molecular building blocks have attracted the attention of scientific community since last couple of decades. The emergence of pathogenic resistance is a natural phenomenon and investigations toward the advances of new structurally diverse inhibitors have always been at the esteem of pharmaceutical research. This growing field has ever become an active area of research for the synthetic, biological and medicinal perspectives, looking at the molecular level. Enzyme inhibition has attracted great attention of biomedical scientist since last couple of decades. A variety of inhibitors have been discovered and used for the control of various diseases. One of the key features of enzyme is its selectivity towards certain inhibitors; the inhibitor can be a simple organic molecule or complex molecular architecture. The specificity of inhibitor depends on the size, shape and the interactive forces which result in the exact matching of inhibitor and the enzyme. These inhibitions block the activity of the enzyme under the physiological conditions. Urea and urease are the landmark molecules in the early days of synthetic organic chemistry. Urea was the first organic molecule synthesized in laboratory and urease was being first crystallized out from jack bean (Amtul et al., 2002; Hagar et al., 1925; Mobley et al., 1989; Amtul et al., 2006; Zonia et al., 1995). The significance of the urease enzyme is critically investigated due to its capacity to serve as a virulence factor of infections in the urinary and gastrointestinal tracts, maintaining equilibrium in the nitrogen cycles of nitrogenous wastes in the rumens of domestic livestock, and its importance in environmental transformations of nitrogenous compounds, including urea based fertilizers. The physiology of microbial ureases depends on their cellular location, regulation, genetic makeup and the relationships between the microbial urease and the well-characterized potent range of inhibitors. The urease (urea amido hydrolase; EC 3.5.1.5) is a nickel containing enzyme which hydrolyzes urea into ammonia and carbamic acid, the later compound produce ammonia and carbon dioxide on further decomposition. The hydrolysis of the urea yields high concentrations of ammonia which accompanying in pH elevation. Urease has been regarded as the important enzyme for the manipulation of urea and other nitrogenous ingredients in the biological, agricultural and environmental fields. Urease occurs in many plants, selected fungi, and a

wide variety of prokaryotes. This enzyme is having important negative implications in medicine, agriculture and environment. The urease inhibitors can play a vital role to counter effect the negative role of urease in living organisms. Urease inhibitors are effective against several serious infections caused by the secretion of urease by Helicobactor pylori which includes gastric tract syndromes, proteus related species in the urinary tract, struvite urolithiasis mainly in dogs and cats. The urease inhibitors have also received great attention from scientists in the soil sciences whereas the nutrient miss-management is associated to the excessive use of synthetic fertilizer and excess of urea products. Research on urease inhibitions yielded several vital therapeutic drugs (Amtul et al., 2002). Hagar and Magath have reported in 1925 that urease is the primary source for the biochemical formation of stone in urinary tract (Hagar et al., 1925). Urease serves as virulence factor in the pathogens responsible for kidney stones entailed in urolithiasis that contributes toward the acute pyelonephritis with other urinary tract infection which induced arthritis and gastric intestinal infections and ultimately the urease imbalance lead to peptic ulcers (Mobley et al., 1989). Urease controls the nitrogen contents in the physiological systems, while it provides a protection mechanism against predators and phytphathogenic organisms (Amtul et al., 2006; Zonia et al., 1995). The high concentrations of ammonia arising from the biochemical reactions of urease, as well as pH elevation result into important negative implications in medicine and agriculture (Zonia et al., 1995; Collins et al., 1993; Montecucco et al 2001; Zhengping et al). High concentrations of ureases cause significant environmental and economic problems by releasing abnormally large amounts of ammonia into the atmosphere during urea fertilization. It induces plant damage primarily by depriving plants of their essential nutrients and secondarily by ammonia toxicity which result in pH increase of the soil (Bremner et al., 1995). The urease inhibitors have played a vital role in the controlling the Helicobacter pylori which caused an imbalance of ammonia levels in cirrhotic patients (Zullo et al., 1998). The urease based liquid crystal-sensors are also used for the detection of heavy metals; these urease based sensors are useful in monitoring the inhibition of enzymatic activities that has been widely investigated for the detection of heavy metals (HMs) (Verma et al., 2005). In many cases, after inhibition of immobilized enzymes with HMs, chelating agents such as EDTA are usually used to regenerate the active site of the enzyme (Bracka et al., 2000). Urease-based sensors, which are inexpensive and sensitive to HMs, have been widely exploited in the scientific community (Volotovsky et al., 1997). Several analytical methods for monitoring HMs are based on the enzyme-catalyzed hydrolysis of urea into ammonia and carbon dioxide, as the consumption of urease inhibitors is vital for the production of grains over the long-term, meanwhile the coating of urea to reduce ammonia volatilization loss from urea fertilizer is gaining great attention of researchers in order to control the depletion of active ingredients in the soil. The use of fertilizer is very much common in the areas where the active ingredients do not occur in sufficient quantities. The nitrogen losses can be reduced in these situations and the use of urease inhibitor is applied to the fertilizer to maintain the optimum pH of the soil (Schwedt et aj., 1993; Saboury et al., 2010). Recently, the LCs has been doped with functional molecules that have been used to develop novel types of sensors. There are series of compounds with 4-pentyl-biphenyl-4-carboxylic acid (PBA), which contains pH-sensitive functional groups could be used in an LC-based pH sensor to monitor small amounts of H^+ released from enzymatic reactions, especially in solutions with a high buffer capacity (Bi et

al., 2009). Literature is rich on the role o*f H. pylori w*hich is the main cause for the gastric infections. Urease is a prominent antigen of the *H. Pylori* which has served as a powerful immunogen for this organism. There have been various reports on large number of patients who have shown gastritis significantly elevated response in immunoglobulins G and A along with urease in the blood serum samples with relative comparison of pre-infected levels. Range of enzymatic assays is reported to measure the immune responses and these are used for the diagnostic purposes in monitoring the antibiotic therapy of *H-Pylori* for the epidemiological studies (Amtul et al., 2002).

2. Inhibition mechanism

The inhibition mechanism is mainly based on the binding of the substrate and enzyme. The urease has a bi metallic centric structure which binds with range of molecules depending on the kinetics, dynamics, mechanism of action and the possible secondary interactions involved in this phenomenon. The mechanism of inhibition can be categorized on the basis of inhibitor, either as irreversible inhibitors or the reversible inhibitor where as the later one is further classified into two categories based on the binding with enzyme either in a competitive manner or in a non competitive manner. The process involved in case of reversible inhibitors mostly occur through non covalent interactions such as hydrogen bonding, hydrophobic interaction and orientation of inhibitor and enzyme in an organized fashion (Amtul et al., 2002). The irreversible inhibitors interact through its functional groups with amino acids in the active site, irreversibly e.g. the nerve gases and pesticides, which contain an organophosphorus that bind with serine residues in the enzyme acetylcholine esterase. The reversible inhibitors are of two types either as competitive or non-competitive. The competitive inhibitors compete with the substrate molecules for the active site while the inhibitor's action is proportional to its concentration that resembles with the substrate's structure closely. The non-competitive inhibitors are not influenced by the concentration of the substrate. It inhibits by binding irreversibly to the enzyme but not at the active site. The non-competitor inhibitor follows the allosteric sites in the enzyme for the binding (Figure 2.1). The reversible inhibitors can be washed out of the solution of enzyme by dialysis.

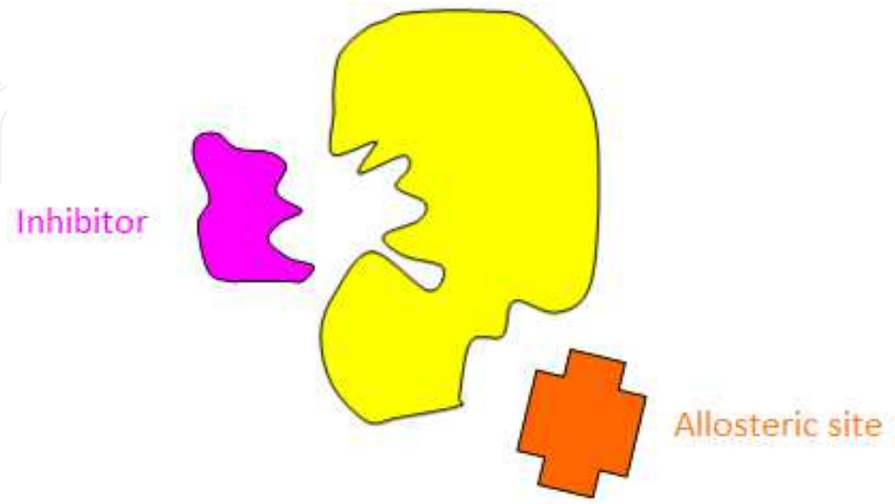


Fig. 1. Model representation of the binding of the enzyme active site and inhibitor.

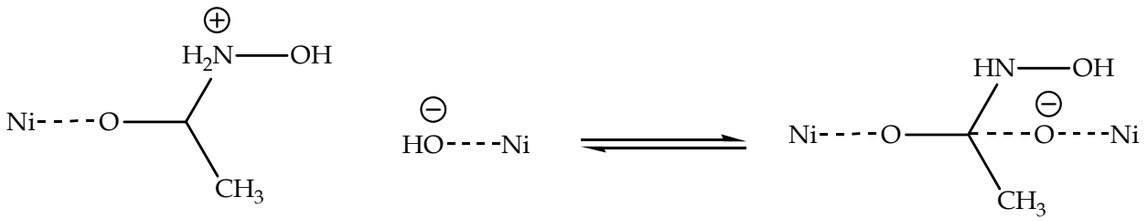
In the competitive inhibition the inhibitor directly binds to the active site of the enzyme *via* a specific host and guest complex, while in the non-competitive mechanism the substrate binds somewhere else and not specifically on the active site in the enzyme this binding of substrate affect the structure of the enzyme and induce inhibition. Furthermore another mode of binding of inhibitor is the un-competitive mechanism, in this mechanism the inhibitor irreversibly binds with the enzyme and result an intermediate complex (E-I*) between the enzyme and the inhibitor. This portion of enzyme is known as allosteric site (Figure 1). The binding of enzyme with the substrate occurs in an organized fashion and the substrates have specific affinity toward the enzyme. Though the active site is a specific region in the enzyme and this specificity depends on the pro-non covalent interaction available for interaction in the inhibitors. These non covalent interactions between the enzyme and inhibitor are the main feature for the chemoselectivity of the substrate and enzymes during formation of the complex.

These interactions mainly provide a surface to regenerate the enzyme for the reaction cycle as a catalyst. The unique geometrical shapes and topology of the active site is complimentary for the substrate molecule, which fit like lock and key arrangement. The enzymes specifically react with only few compounds of the similar structural features, these structural feature in the inhibitor is known as pharmacohores, while the potency of the inhibitor is managed by appending various functional groups to the main skeleton of the inhibitor by making target oriented derivative containing these features and consequently resulted in the controlled target oriented synthesis of the potent and fascinating inhibitors. As there are so many theories which explain the mechanistic perspective of the enzymatic action, only the 'lock and key' and the 'induced fit' theories are well accepted (Donald et al., 2005). The mechanistic studies of the biological systems undergoes *via* a complex phenomenon, a large number of literature reports are available involving the structure-based design and testing of novel pharmacophores model for the recognition of urease inhibitors. The intimated details of the molecular geometry of urease enzyme as well as its mechanism of urea hydrolysis have been confirmed with XRD-analysis.

2.1 Kinetics of enzyme activity

The rate of reaction provide a clue about the affinity of the inhibitor towards the enzyme, while at the same time it also effect the potency of the inhibitor. The kinetic effect of rebeprazole has been investigated on the urease inhibition, it is reported that rebeprazole act as an irreversible noncompetitive inhibitor. Mainly the associated inhibitory potency of rabeprazole is dependent on the pH of reaction mixture and K_i value which varies at the progressive inactivation of urease by rabeprazole, initially it proceeds according to pseudo-first-order kinetics with respect to the remaining enzymatic activity at pH 7.0 and 37 °C, with a second-order rate constant of 0.0017 μM^{-1} s^{-1}. This inhibitor competes with substrate to bind to the enzyme and form as an E-I (enzyme and inhibitor) complex, that slowly transformed to an E-I* complex which is stable (Scheme 1) (Park et al., 1996).

The use of non-steady-state kinetic measurements is important to properly analyze the non competitive inhibition (Mobley et al., 1989; Rosenstein et al, 1984). A number of mechanistic studies have been carried out on various inhibitors. It is reported that hydroxamic acid which suggests that a bidentate complex form with one of the nickel ions, quite few scientists



E.I E.I*

E.I = Enzyme-Inhibitor Complex
E.I* = Stable Enzyme-Inhibitor Complex

Scheme 1. Bi-dentate ligand complex of substrate and enzyme.

believe that it make a bridge between the two nickle ions. Similarly it is also suggested that the E-I and E-I* type of interactions occurs, forming an initial monodentate species that bridges the two nickel ions at the active site (Amtul et al., 2002). The non-steady-state studies have also been employed with other competitive inhibitors, the specific examples for the kinetic analysis is the interaction of phenylphosphoro diamidate with urease (*K. aerogenes*) with dissociation constant of 4.7×10^{-5} s^{-1}. The proposed studies suggests that the E-I state where an inhibitor bound in unidentate mode to one nickel ion while the EI* species involves the bridging of the two nickel ion with inhibitor where R is the aromatic ring *via* the formation of tetrahedral intermediate rather that the hypothetical trigonal bipyramidyl intermediate. Here the hydrolysis occurs with simple in-line displacement (Andrews et al., 1984).

2.2 Action of inhibition

The modes of action of enzyme have been investigated on the thiol based compounds. As the presence of an auxochrome sulphar (thio) moiety in the inhibitor help in characterization *via* UV-Visible spectroscopy for a variety of enzymes *e. g* jack bean and *K aerogenes* ureases, these spectroscopic perturbation is consistent with the development of thiolate. It is well documented that the Ni (II) charge-transfer complex is kinetically controlled process. Since a competition of thiol with urea for the active sites of the enzyme in binding the nickel provide a relative reference to further study the action of inhibitor over the enzyme.

A B

Scheme 2. Structural hypothesis for the competitive interaction of the urease active sites with thiourea (A) and hydroxyurea (B).

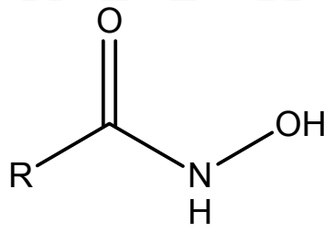
The mechanism of action related to hydrolysis of urease to ammonia and carbamate has been investigated in depth, the later compound spontaneously decomposes to the carbamic acid, accompanying the deprotonation generating equilibrium as a result of increase in pH. The research on urease has contributed extensively towards development of medicinal organic chemistry (Summer et al., 1926) and crystallization (Amtul et al., 2002). The urea binds into the pocked adjacent to Ni near Wat-502. The H2-H4 mobile flap may open to allow access into the active site of enzyme, once the active functional group binds to Ni-1 *via* coordination. The urea is then placed in a manner that allows the coordination of its oxygen with the N epsilon of His 219, by forming hydrogen bond. The urea also accepts a hydrogen bond from the interaction of its amide nitrogen with side chain of His 320. These non covalent interactions are oriented to urea in an organized fashion so that the carbonyl group of the urea undergoes polarization and attacked by hydroxide (Wat-502), that coordinates other metal atom of Ni-2 and forms tetrahedral intermediate. As one proton is transferred from His-320 to the nitrogen (leaving group), meanwhile the intermediate collapses into ammonia and carbamate. The carbamate undergoes hydrolysis to form carbamic acid and second molecule of ammonia (Amtul et al., 2002). The suboptimal interactions provide a significant source of substrate binding energy; this energy is commonly found in the enzymes with mobile active site loops which undergo induced fit. The side chain of His 320 in urease is in close proximity to the side chain of Asp 221 and Arg 336, whereas the geometry is not optimal for the hydrogen bonding between the residues. His 320 also interacts with Wat-170 *via* hydrogen bonding. The Wat-170 also forms hydrogen bond with two main chain carbonyls. Wat-170 does not have the ability to donate three hydrogen bonds, so one of the H-bond at any given time is unable to form, making the interactions of water suboptimal. There are also suboptimal interactions between the nickel metallo center and solvent. The active site water molecules (Wat-500, Wat, 501, Wat-502) have inter-oxygen distance that is too short to allow for simultaneous occupancy. Since the occupancy of the active site is too small to accommodate all three water molecules, there appears a competition for occupancy at the three positions. All these interactions must contribute to the flexibility of the mobile flap by the destabilizing the closed positions. These interactions are the main driving force for the catalytic mechanism of the urease in the various biological processes. The most of the biochemical reactions occurs by decreasing the free energy difference between the unbound enzyme with substrate and enzyme-substrate complex which accomplished in two ways with enzyme binding transition state which is highly favorable or the initial state which has higher free energy, possibly the addition of suboptimal interactions. Such interaction makes possible transition state which usually is not favorable, therefore most of the urease mechanisms involve a number of suboptimal interactions to derive the hydrolysis of urea in the systems (Mobley et al., 1989; Mulvaney et al., 1981).

3. Potent inhibitors

A vast range of naturally isolated and synthetic urease inhibitors has been reported in the literature though pharmacological importance and the developed resistance in the bacterial infections have attracted the attention of the scientific community to explore new versatile urease inhibitors.

Urease inhibitors are classified broadly into two major classes as metal-organic and organic compounds, the later examples are such as acetohydroxamic acid, humic acid and 1,4-

benzoquinone etc. The inhibitors have broad applications in combination with urea fertilizers that has been proposed for soil urease activity control (Bundy et al, 1973; Amtul et al., 2002). There is an example of kinetic studies on quinone-induced inhibition of urease has also been investigated, one among those is polyhalogenated benzoand naphthoquinones, in which the quinones were found to be non-competitive inhibitors of jack bean and bacterial ureases of different strengths (Zaborska et al., 2002; Pearson et al., 1997). Several other potent inhibitors are being used as first line of treatment for infections causes by urease-producing bacteria which are available in current market. The most effective inhibitors with safe great potency profile for considerable control of urease-related ailments are still needed. The available data provide a basis for urease inhibition properties of thiol-compounds, different cysteine derivatives (CysDs) with cysteine-like scaffold arylidene barbiturates have been studied as urease inhibitors such as *N*-Substituted Hydroxyureas (Amtul et al., 2002; Amtul et al., 2004; Todd et al., 1989). Acetohydroxamates are the bacterial urease inhibitor and used as therapeutic potential in hyperammonaemic states (Carlini et al., 2002). The urease inhibitors have also been employed for the coating of urea in the agricultural soils to reduce the ammonia mitigating. The nitrogen loss depends on the rate of biodegradation and constant volatilization in agricultural soils of urea fertilizer, such coating can improve the bioavailability of nitrogen and consequently increase dry matter yield and nitrogen uptake. These increases of urea contents in soil result from delayed urea hydrolysis by urease inhibitors and coating materials. The value of inhibitors in nitrogen mitigating is depending on the rate of biodegradation and persistence in soils (Amtul et al., 2007). The urease inhibitors have been investigated for the treatment of bacterial infections and also for the excessive urea break down in soil, therefore studies on the potent and specific inhibitors have been an active area of research. Taking in view the great potential of urease in medicine and soil sciences, several classes of urease inhibitor have been discovered in the recent years (Ara et al., 2007; Mobley et al., 1989; Mulvaney et al, 1981; Rosenstein et al., 1984). The urease inhibitors can also be classified into two categories either as metal complexes or organic compounds. The organic compounds are further classified into the three main classes such as hydroxamic acid analogs, phosphoramide compounds and thiourea derivatives. Hydroxamic acid (Scheme 3) analogs are the most commonly known urease inhibitors and first member of its series was hydroxamic acid which was discovered in 1962. The acetohydraxamic acid with other numerous derivatives have been synthesized and studied against ureases of plants and bacterial origin (Mobley et al., 1989). The other members of the series include *n*-aliphatic hydroxamic acid, ortho or para substituted benzohydroxamic acid, *trans*-cinnamoyl hydroxamic acid with other functional groups and substituents.



Scheme 3. General structure of hydroxamic acid.

Phosphoramide compounds are another class of potent inhibitors of the urease enzyme, this class of inhibitors comprise of a large number of simple and complex compounds. Mainly

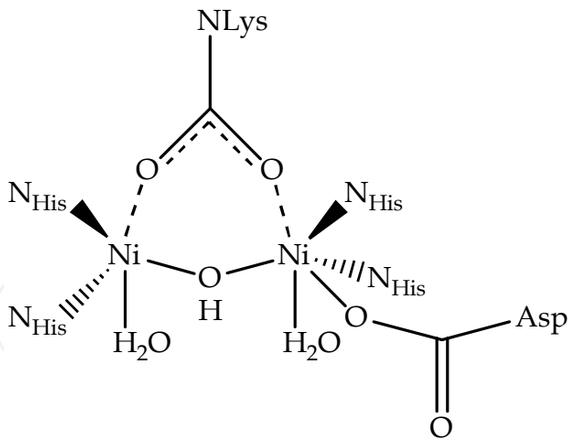
the less complex examples includes of phosphoramidates and diamidophos phate with substituted phenylphosphoradiamidates, while a range of N-acyl phosphoric triamidates (Humayun et al., 2010).

Thio substituted compounds are also strong inhibitors of the urease enzyme. This includes cysteamine containing a cationic β-amino cysteamine, which exhibit highest affinity for the urease, on the other hand the thiolates containing anionic carboxyl groups are uniformly poor inhibitors. The pH dependence studies demonstrate that the actual inhibitor is the thiolate anion (Ansari et al., 2005). A series of bezothiazepines have also been investigated bearing significant urease inhibitory activities (Kühler et al., 1998). The studies on structure activity relationship of the thio substituted benzimidazoles with *in-vitro* and *in-vivo* efficacy model, where the potency of the substrate is depend on the substituents positions in the pharmacophore (Creason et al., 1990). The inhibitory effects of *N-(n*-butyl) thiophosphora-triamides (NBPT) and its oxon analogs in the soil are also been reported (Zaborska et al., 2004). There are several other potent inhibitors readily available in the market the most common is NBPT (N-[n-butyl] thiophosphoratriamide) which is sold worldwide under the trade name of Agrotain. This fertilizer has amended the coats of urea preventing the urease enzyme from breaking down the urea for up to fourteen days of life. As this increases the probability that urea will be absorbed into the soil after a rain rather than volatilized into the atmosphere which reduce the nitrogen content in the soil. The rich content of urea in soils causes hydroxylation under the surface of soil and decreases atmospheric losses of the major ingredients of fertilizers. The use of potent inhibitors also decreases the localized zones of high pH which is common in untreated urea agricultural fields. These remedies are commonly employed to maintain the pH of the soil and controlled ammonia volatilization. The use of inhibitors increases the efficiency of urea in delivering nitrogen to soil.

A wide range of metal complexes with meager to good urease inhibitory activities are reported which includes heavy metal ions, such as Cu^{2+}, Zn^{2+}, Pd^{2+} and Cd^{2+} (Zaborska et al., 2001; Asato et al., 1997). There are reports on the urease inhibitory activities of organotin(IV), vanadium(IV), bismuth(III), copper(II), and cadmium(II) complexes (Khan et al., 2007; Ara et al., 2007; Hou et al., 2008; You et al., 2008; Tanaka et al., 2003; Cheng et al., 2007; You et al., 2010). A series of metal complexes of Schiff bases such as thiocyanato-coordinated manganese(III) complexes based on N-ethylethane-1,2-diamine and N-(2-hydroxyethyl)ethane-1,2-diamine have shown significant urease (Huang et al., 2011). There is library of compounds are available in various databases and the recent developments is continuously adding numerous class of of hetero-dinuclear CuII–ZnII complexes (You et al., 2011).

4. Structure Activity Relations (SAR)

The hydroxamic is the most commonly used urease inhibitor coordinating to nickel of urease through carboxylic acid and hydroxyl amine (Scheme 3). The structural studies on the urease from *Klebsiella aerogenes*, *Bacillus pasteurii,* and *Helicobacter pylori* have revealed that it contains a dinuclear Ni active site with a modified amino acid side chain-containing a carbamylated lysine residue that bridges the deeply buried metal atoms as shown in Scheme 3 (Benini et al., 1999; Jabri et al., 1995; Ha et al., 2001). The crystal structure has been resolved only for bacterial ureases; it has an active centre which contains two simple coordinated water molecules and a bridging OH group. The specificity of the enzyme is closely related to the shape of its active centre (Mobley et al., 1995).



Scheme 4. Active center of urease.

There is vast reports on the structure of urease, the *Klebsiella areogenes,* the urease structure has a trimer of alpha, beta and gamma units in a triangular arrangement with three active sites per enzyme. These subunits include a 60.3 KD apha, a 11.7 KD beta and a 11.1 KD gamma subunit (Jabr et al., 1992). The interactions between the individual subunits of all three α subunits make extensive contacts with each other to build a trimer. Each alpha subunit packs between two beta and two gamma subunits to form the side of the triangle arrangement (Jabri et al., 1995). Each beta subunit packs between the two of the adjacent alpha subunits at each corners of the triangle. The remaining gamma subunit interacts with two gamma subunits at the crystallographic three fold axis. Because of the high degree of interaction, it is not obvious that which alpha, beta and gamma chains make up the primary units. High conservation of sequences and the extensive interactions of the trimer suggest a similar trimer structure in all the urease which is further subdivided into sub-units. The gamma subunit consists of an alpha-beta domain with four helices and two anti parallel stands. Two helices (b and c) and the two strands pack together with right-handed up-down-up-down topological order. One of the remaining helices (a) is lined up peripherally and interacts at the three fold axis with the other two gamma subunits, tightly packing against itself and to other (a) helices in an orthogonal manner. The last helix (d) experience single turn. The first fifteen residues of the beta subunits form two antiparallels beta sheets with the amino-terminal residues of the alpha subunits. The core of the beta subunit forms an imperfect six-stranded antiparallel beta sheet (Mobley et al., 1989; Hausinger et al., 1993). This subunit stabilizes the trimer by interacting with domain two of its own alpha subunits and domain one of a symmetry related alpha subunit. The alpha subunit consists of two domains, an (alpha, beta) 8 barrel domain and a primarily beta domain. The (alpha, beta) 8 barrel is the only domain that contributes to the active site. Its barrel is rather elliptical with the long axis connecting strands 1 and 7. Strands 9 and 10 extend the lower portion of strands 1 and 3. The active site is located at the carboxyl terminal of the strands. A flap is formed that covers the active site by a helical excursion between strand 7 and helix 7. Mixed four-strands and eight-stranded beta sheets form the wall of U-Shaped canyon which makes up the second beta domain. The walls are connected by the long strands 5 and 6 together with strands 10 and 11 which go down on one side, and up on the other side. The structure activity relationship of the various urease inhibitors are reported in literature, the studies on *in-vitro* with *in-vivo* efficacy of the effect benzimidazoles suggested that the orientation and position of the pyridyl moiety larger and more lipophillic chromophore of the

substrate has affinity which affect the minimum bacterial concentration of the helicobacter pylori (Kühler et al., 1998).

5. Conclusion

The chapter is focused on the importance and future implications of the urease. The emphases have been laid on urease keeping in mind its role as virulence factor for the urinary tract infections and gastrointestinal infections while the inhibition of urease is also effective in the agricultural sector. Urease enzyme inhibition has become a growing area of research at the interface of the biomedical sciences. The broad range of potent inhibitors has been reported in literature from simple organic molecules to complex molecular building block with metal complexes. These molecular building blocks are expected to bring revolution in pharmaceutical, agricultural and environmental fields. Urease based sensors, adsorbent and other devices have also been discussed that are commercially used.

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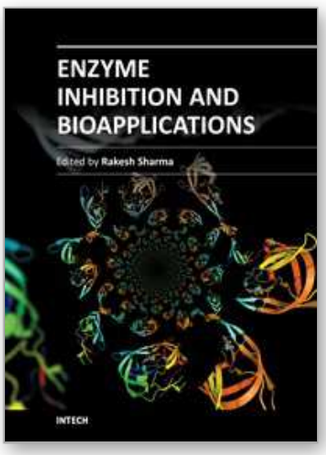

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Enzyme Inhibition and Bioapplications is a concise book on applied methods of enzymes used in drug testing. The present volume will serve the purpose of applied drug evaluation methods in research projects, as well as relatively experienced enzyme scientists who might wish to develop their experiments further. Chapters are arranged in the order of basic concepts of enzyme inhibition and physiological basis of cytochromes followed by new concepts of applied drug therapy; reliability analysis; and new enzyme applications from mechanistic point of view.

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51000 Rijeka, Croatia
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