The π-Helix Formation between Asp^{369} and Thr^{375} as a Key Factor in E_1-E_2 Conformational Change of Na^+/K^+-ATPase

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Short title: Structure of Na^+/K^+-ATPase in E_1-E_2 conformational change

Summary

Molecular modeling of the H_4-H_5-loop of the α_2 isoform of Na^+/K^+-ATPase in the E_1 and E_2 conformations revealed that twisting of the nucleotide (N) domain toward the phosphorylation (P) domain is connected with the formation of a short π-helix between Asp^{369} and Thr^{375}. This conformational change close to the hinge region between the N-domain part of the P-domain could be an important event leading to a bending of the N-domain by 64.7° and to a shortening of the distance between the ATP binding site and the phosphorylation site (Asp^{369}) by 1.22 nm from 3.22 nm to 2.00 nm. It is hypothesized that this shortening mechanism is involved in the Na^+-dependent formation of the Asp^{369} phospho-intermediate as part of the overall Na^+/K^+-ATPase activity.

Key words

Computer modeling • Molecular dynamics simulations • Molecular structure • Na^+/K^+-ATPase • E_1/E_2 conformation

Introduction

Na^+/K^+-ATPase (EC 3.6.1.37) transports Na^+ and K^+ ions against an electrochemical gradient across the plasma membrane (Skou 1957). Na^+/K^+ transport by the sodium pump is intimately connected to conformational changes of the α-subunit (≈ 110 000 Da) of Na^+/K^+-ATPase and is energized by ATP. ATP hydrolysis proceeds via a Na^+-dependent formation of an aspartyl^{369} phospho-intermediate and a K^+-activated hydrolysis of it (Lutsenko and Kaplan 1995, Kaplan 2002). Analogous processes occur in other P-type ATPases, such as H^+/K^+-ATPase and Ca^{2+} -ATPase of sarcoplasmic reticulum (Kaplan 2002, Toyoshima and Inesi 2004). The three-dimensional (3D) structure of the latter enzyme has been elucidated in several conformational

states (Toyoshima and Inesi 2004). Structural 3D information of Na^+/K^+-ATPase, however, is relatively vague. Consistent with the long distance of about 3 nm between the ATP binding site at the N-domain and Asp^{369} in the E_1 conformation (Ettrich *et al.* 2001, Tejral *et al.* 2007), the various preparations of the isolated H_4-H_5-loop can bind ATP only but not hydrolyze it, or to phosphorylate the phosphorylation site (Gatto *et al.* 1998, Costa *et al.* 2003). An intrinsic *p*-nitrophenylphosphatase of these preparations (Tran and Farley 1999) is unrelated to the catalytic activities of the whole enzyme (Krumscheid *et al.* 2004).

An attractive option to understand the so-called hinge movement, i.e. how the distance between N and P domains of the α-subunit is shortened, is computer modeling based on homology and similarity with proteins of known structures. Since the high resolution 3D structures of the Ca^{2+}-ATPase pump from sarcoplasmic reticulum in both E_1 and E_2 conformations are known (Toyoshima *et al.* 2000, Toyoshima and Nomura 2002), it is possible to deduce by restraint-based comparative modeling the analogous 3D structure of the H_4-H_5-loop of Na^+/K^+-ATPase which has about 30 % identity and 65 % similarity with Ca^{2+}-ATPase.

We computed the 3D model of the H_4-H_5-loop of the α_1 isoform of Na^+/K^+-ATPase α-subunit from pig kidney in E_1 conformation between Leu^{354} and Leu^{773} (Ettrich *et al.* 2001) and verified the developed model using a combination of advanced biophysical and molecular biology techniques (Kubala *et al.* 2002, Krumscheid *et al.* 2003, Kubala *et al.* 2003, Krumscheid *et al.* 2004, Lansky *et al.* 2004). We also demonstrated that eight amino acid residues (Glu^{446}, Phe^{475}, Lys^{480}, Gln^{482}, Lys^{500}, Gly^{501}, Phe^{547}, Cys^{548}) in the N-domain form the ATP-binding site and that the shape of the pocket is stabilized by a hydrogen bond between Arg^{423} and Glu^{472} (Lansky *et al.* 2004). Recently, we also computed a 3D model of the H_4-H_5-loop of Na^+/K^+-ATPase α_2 isoform from mouse brain between amino acid residues Cys^{336} and Arg^{758} in its E_1 conformation (Tejral *et al.* 2007).

However, the molecular mechanism of the enzyme phosphorylation and phosphate transfer from the ATP-binding site to the phosphorylation site remain little known. To understand the overall catalytic process on a molecular level, detailed 3D structures of Na^+/K^+-ATPase H_4-H_5-loop conformations must be determined. This paper is focused on the determination of the structure of the H_4-H_5-loop of the Na^+/K^+-ATPase α_2 isoform in E_2 conformation and compares it with the structure in the E_1 conformation.

Methods

The model was created using free software and developed as described previously (Tejral *et al.* 2007). The Q3UHK5 sequence in the length of 1020 amino acids for the Na^+/K^+-ATPase α_2 isoform subunit precursor from mouse brain (*Mus musculus*, adult male brain) was chosen.

After the transmembrane helix topology prediction in the MEMSAT program (Jones *et al.* 1994, Jones 1998), a rough secondary structure based on protein sequence was predicted using the PSIPRED program (Jones 1999a, McGuffin *et al.* 2000, Bryson *et al.* 2005). The resulting H_4-H_5-loop target sequence Cys^{336}–Arg^{758} was determined. Appropriate templates were found using GenTHREADER (Jones 1999b, McGuffin and Jones 2003). For modeling of the E_1 conformation of the H_4-H_5-loop (Tejral *et al.* 2007), the structures 1Q3I (Hakansson 2003), 1MO7 (Hilge *et al.* 2003), 1SU4 (Toyoshima *et al.* 2000) and of the E_2 conformation 1Q3I (Hakansson 2003), 1MO8 (Hilge *et al.* 2003), 1KJU (Xu *et al.* 2002) templates were used.

The multialignments of target sequence and templates chosen for particular conformation was prepared in the ClustalX program (Thompson *et al.* 1997) and is described in a previous paper (Tejral *et al.* 2007). The model in both E_1 and E_2 conformations was generated in MODELLER (Sali and Blundell 1993, Fiser *et al.* 2000). For modeling, additional spatial restraints were introduced; for details, see (Tejral *et al.* 2007). Individual secondary structures

arising from generated 3D models of the H_4-H_5-loop were localized using the DSSP program (Kabsch and Sander 1983). The calculated model was verified using the PROCHECK (Morris *et al.* 1992, Laskowski *et al.* 1993) and Verifi3D programs (Bowie *et al.* 1991, Luthy *et al.* 1992). The main E_1-E_2 conformational transition changes were determined with the Dyndom program (Hayward and Lee 2002). The native structure of the H_4-H_5-loop was created in GROMACS suit of programs (Lindahl *et al.* 2001, Van der Spoel *et al.* 2005). The water cubic box with periodic binding conditions and minimum distance of 1.5 nm between the solute and the box and with the SPC model for water molecules (Eisenberg and McLachlan 1986) utilizing force field G43a1 were used. The water-box energy was minimized by means of conjugate-gradient method with the tolerance of 100 $kJ.mol^{-1}.nm^{-1}$.

Results

Three-dimensional model of the H_4-H_5-loop of Na^+/K^+-ATPase in E_1 and E_2 conformations

Using restraint-based comparative modeling, structures of the H_4-H_5-loop of Na^+/K^+-ATPase from mouse brain (*Mus musculus*, adult male brain, α_2 isoform) between Cys^{336} and Arg^{758} in both E_1 and E_2 conformations were calculated. Notably, the new 3D model was constructed based not only on similarity with Ca^{2+}-ATPase , but also on known structures of Na^+/K^+-ATPase segments. The 1Q3I sequence (Hakansson 2003) is porcine (*Sus scrofa*) N-domain Na^+/K^+-ATPase α_2 isoform crystal structure. 1MO7 and 1MO8 structures (Hilge *et al.* 2003) represent the N-domain of Na^+/K^+-ATPase α_1 isoform from rat (*Rattus norvegicus)* determined by high resolution NMR – the 1MO7 in E_1 and 1MO8 E_2 conformation. The 1SU4 sequence represents the crystal structure at 2.4 Å resolution of the Ca^{2+}-ATPase from sarcoplasmic reticulum (SERCA1a) of rabbit hind leg muscle (*Oryctolagus cuniculus*, skeletal muscle) in the E_1 conformation (Toyoshima *et al.* 2000); the 1KJU Ca^{2+}-ATPase E_2

conformation structure (Xu *et al.* 2002) was determined by cryoelectron microscopy. Structures without any bound ions were used only.

The calculated structure (Fig. 1) clearly suggests no significant differences between the α_2 isoform of mouse brain (between Cys^{336} and Arg^{758}) and the α_1 isoform of pig kidney (between Leu^{354} and Leu^{773}) (Ettrich *et al.* 2001) of the H_4-H_5-loop of Na^+/K^+-ATPase in E_1 conformation (Fig. 1A). The structure consists of two well separated parts. The N-domain is formed by a seven-stranded antiparallel β-sheet with two additional β-strands and five α-helices sandwiching it. The ATP-binding site localized on the N-domain was found to be identical in both α_2- and α_1-isoforms. The P-domain comprises a typical Rossman fold. The P-domain is created by amino acid residues of the two sub-domains (Lys^{347}–Asn^{377} and Ala^{589}–Asn^{746}) clearly separated from the N-domain (Arg^{378}–Arg^{588}). The phosphorylation site with Asp^{369} phosphorylation residue is localized in the central part of the P-domain, located at the C-terminal end of the central β-sheet. The calculated E_1 structure is in good agreement with our previous model (Ettrich *et al.* 2001) as well as with published NMR and crystal structures of the Na^+/K^+-ATPase N-domain (Hakansson 2003, Hilge *et al.* 2003). Moreover, our transmembrane segment determination is in agreement with published data (Purhonen *et al.* 2006).

The main objective, however, was to analyze the enzyme E_1-E_2 conformational transition. The calculated structures of the H_4-H_5-loop of mouse brain enzyme H_4-H_5-loop between Cys^{336} and Arg^{758} in E_1 and E_2 conformations, presented in Fig. 1A, B, demonstrate clearly structural changes upon this conformational transition. Obviously, a significant part of the H_4-H_5-loop underwent an important structural change. While the N-domain remained virtually conserved during the conformational transition, the P-domain changed considerably. Notably, the most striking difference between the E_1 and E_2 conformations is visible in the hinge region between the N- and the P-domain. Structural changes are important for twisting of the N-domain toward

the P-domain and for shortening of the distance between the α-carbons of Asp^{369} and Phe^{475} from 3.22 to 2.00 nm and also in bending of the N-domain by 64.7°.

Structure of the phosphorylation site of Na^+/K^+-ATPase in E_1 and E_2 conformations

Additional structural differences are also detectable in the P-domain, namely at the phosphorylation site. Details of the 3D structure of the phosphorylation site in both main conformations (Figs. 2A, B and 3A, B) show the P-domain to be composed of seven-stranded parallel β-sheets with eight short α-helices forming a typical Rossman fold flanked by one antiparallel β-strand. A hydrogen bond between the O atom of Asp^{369} and the N atom of Lys^{690} in the E_1 conformation (Fig. 2) disappears in the E_2 conformation (Fig. 3). This hydrogen bond breakage is due to the formation of so-called π-helical structure (Pauling *et al.* 1951) between the amino acid residues Asp^{369} and Thr^{375} in the E_2 conformation. Clearly, Thr^{375} and six amino acid residues between Asp^{369} and Thr^{375} play a key role in the E_1-E_2 conformational transition and formation of the phosphorylation site structure in the E_2 conformation. This π-helix formation is strengthened by repositioning of the hydrogen bond between the O atom of Thr^{375} and the N atom of Gly^{372} as well as the hydrogen bond formation between the N atom of Leu^{374} and the O atom of Lys^{370}, or the N atom of Leu^{374} and the O atom of Asp^{369}. Thr^{375} is localized just on the interface between the N-and P-domains.

Discussion

The 3D model of the H_4-H_5-loop of Na^+/K^+-ATPase from mouse brain (*Mus musculus*, adult male brain, α_2 isoform) between Cys^{336} and Arg^{758} in both E_1 and E_2 conformations was calculated. Modeling approach and comparison with the previous E_1 model (Ettrich *et al.* 2001)

have been discussed elsewhere in detail (Tejral *et al.* 2007). The new model clearly revealed that neither the effect of the amino acid residues localized between Cys^{336} and Leu^{354}, nor the different subtype influence the 3D structure of the H_4-H_5-loop of Na^+/K^+-ATPase.

The modeling approach, utilizing template structures without any bound ions and other substances, resulted in the model enabling further investigation in the sense of docking particular substrates and cofactors and applying molecular dynamics approach to hydrated structures. The docking procedure can amount to undisputable advantages of the comparative modeling approach, allowing investigation of structures not obtainable in laboratory conditions (our previous unpublished data) and comparing the results with available crystallographic data. According to the best of our knowledge, this is the first description of the 3D E_2 conformation of the H_4-H_5-loop of Na^+/K^+-ATPase in such detail.

Molecular modeling of the E_1 and E_2 conformational states of the H_4-H_5-loop of the α_2 subunits of Na^+/K^+-ATPase from mouse brain and their comparison revealed two important structural aspects of this essential step for the Na^+/K^+-activated ATP hydrolysis: (1) The 3D structure of the N-domain remains conserved and hence rather rigid. (2) There are significant conformational changes between the N-terminal and C-terminal subdomains of the P-domain taking place when the enzyme converts from the E_1 to the E_2 conformational state.

The main and the most striking change between the E_1 and E_2 conformations is the bending of the N-domain toward the P-domain by 64.7° (see Fig.1A, B). This rather huge movement is very probably the first inevitable step in the molecular mechanism of the enzyme phosphorylation, a process seen also in Ca^{2+}-ATPase (Toyoshima and Mizutani 2004). Notably, such a bending brings the ATP-binding and phosphorylation sites closely together. The distance between the α-carbon of Phe^{475} at the ATP-binding site and the α-carbon of Asp^{369} at the phosphorylation site decreases by 1.22 nm after E_1-E_2 conformational transition: from 3.22 nm in

the E_1 conformation (Fig. 1A) to 2.00 nm in the E_2 conformation (Fig. 1B). With regard to the distance of 2.00 nm in the E_2 conformation, ATP length of 1.00–1.50 nm depending on the temperature motion, and Asp^{369} and Phe^{475} sidebones total length of about 0.5 nm, mutual interaction of the ATP γ-phosphate with the phosphorylation site is possible. The aromatic ring of Phe^{475} and the adenine ring of ATP are probably parallel and a stacking interaction between their π-electron systems is important for the stabilization of ATP within the binding pocket (Ettrich *et al.* 2001, Kubala *et al.* 2003).

The second key point in the phosphorylation process seems to be the conformational change of the P-domain. Interestingly, the basic structure of the P-domain remains conserved during the E_1-E_2 conformational transition. A rather significant difference, however, occurs in the structure of the phosphorylation site. The phosphorylation site (Asp^{369}) has already been described as ^{369}DKTGS/T^{373} motif (Lutsenko and Kaplan 1995, Kaplan 2002, Scheiner-Bobis 2002, Horisberger 2004). This motif is very important for the phosphate-group coordination. It is well suited to bind both Mg^{2+} as well as the negatively charged phosphate ion. The short helical structure and the hydrogen bond between Asp^{369} and Lys^{690} seem to play a key role in the conformational change (Figs. 2 and 3). The postulated hydrogen bond (Patchornik *et al.* 2000, Jorgensen *et al.* 2003) between the O atom of Asp^{369} and the N atom of Lys^{690} is clearly visible in the E_1 conformation but disappears in the E_2 conformation. This break of a hydrogen bond is apparently followed by the formation of the short π-helix between Asp^{369} and Thr^{375} in the E_2 conformation which is surprisingly missing in the E_1 conformation. This π-helix formation is strengthened by the hydrogen bonds between the N atom of Leu^{374} and the O atom of Lys^{370} or the N atom of Leu^{374} and the O atom of Asp^{369}. Notably, Thr^{375} is localized just on the interface between the N-and P-domains. Consequently, repositioning of Thr^{375} and the rearrangement of

the hydrogen bond between the O atom of Thr^{375} and the N atom of Gly^{372} from the O atom of Thr^{375} in the protein backbone to the O atom of Thr^{375} in the protein sidebone resulting in a change of the Thr^{375} sidebone and the formation of a helical structure at the phosphorylation site in the E_2 conformation are closely connected with the bending of the N-domain. The N-domain bending toward the P-domain resulting finally in the formation of a closed E_2 conformation is also accompanied by N-domain twisting. The angle between the two domains decreases by about 20°. Such a structure is, thus, suitable for phosphate coordination and leads to enzyme phosphorylation.

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Figure legends

Fig. 1. The structure of Na^+/K^+-ATPase from mouse brain (α_2 isoform) in E_1 (A) and E_2 (B) conformations.

The N-domain bent toward the P-domain by 64.8°.

Fig. 2. The structure of Na^+/K^+-ATPase from mouse brain (α_2 isoform) phosphorylation site in the E_1 conformation.

A. and B. represent different perspectives, the viewpoints in 2 and 3 are identical. A hydrogen bond between the O atom of Asp^{369} and the N atom of Lys^{690} detected in the E_1 conformation disappeared in the E_2 conformation (see Fig. 3A, B).

Fig. 3. The structure of Na^+/K^+-ATPase from mouse brain (α_2 isoform) phosphorylation site in the E_2 conformation.

A. and B. represent different perspectives, the viewpoints in 2 and 3 are identical. A hydrogen bond between the O atom of Asp^{369} and the N atom of Lys^{690} detected in the E_1 conformation (see Fig. 2A, B) disappeared in the E_2 conformation accompanied by appearance of the short π-helix. Repositioning of Thr^{375} and the rearrangement of the hydrogen bond between the O atom of Thr^{375} and the N atom of Gly^{372} from the O atom of Thr^{375} in the protein backbone to the O atom of Thr^{375} in the protein sidebone resulting in a change of the Thr^{375} sidebone position and in the formation of a helical structure at the phosphorylation site in E_2 conformation are closely connected with bending of the N-domain.

Figure 1

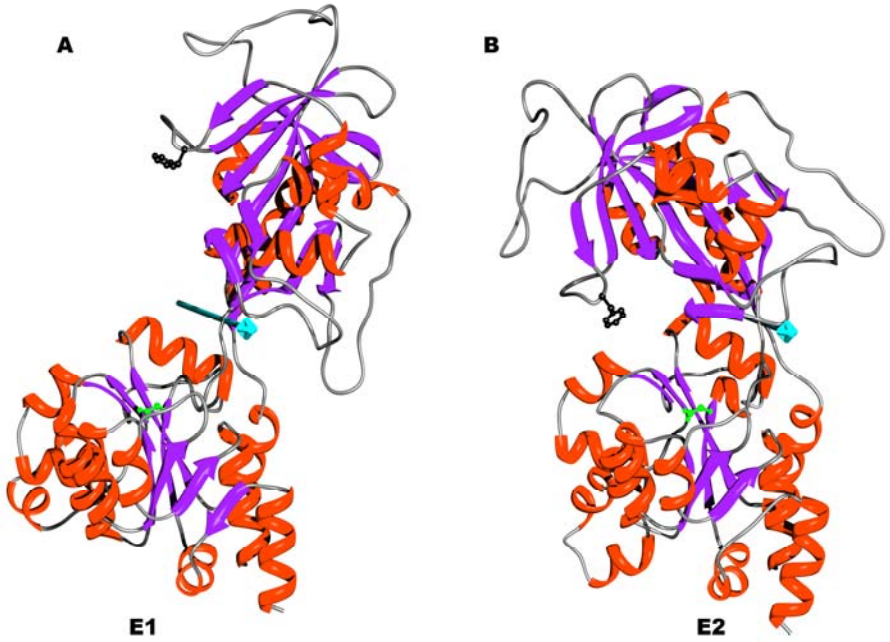

Figure 2A

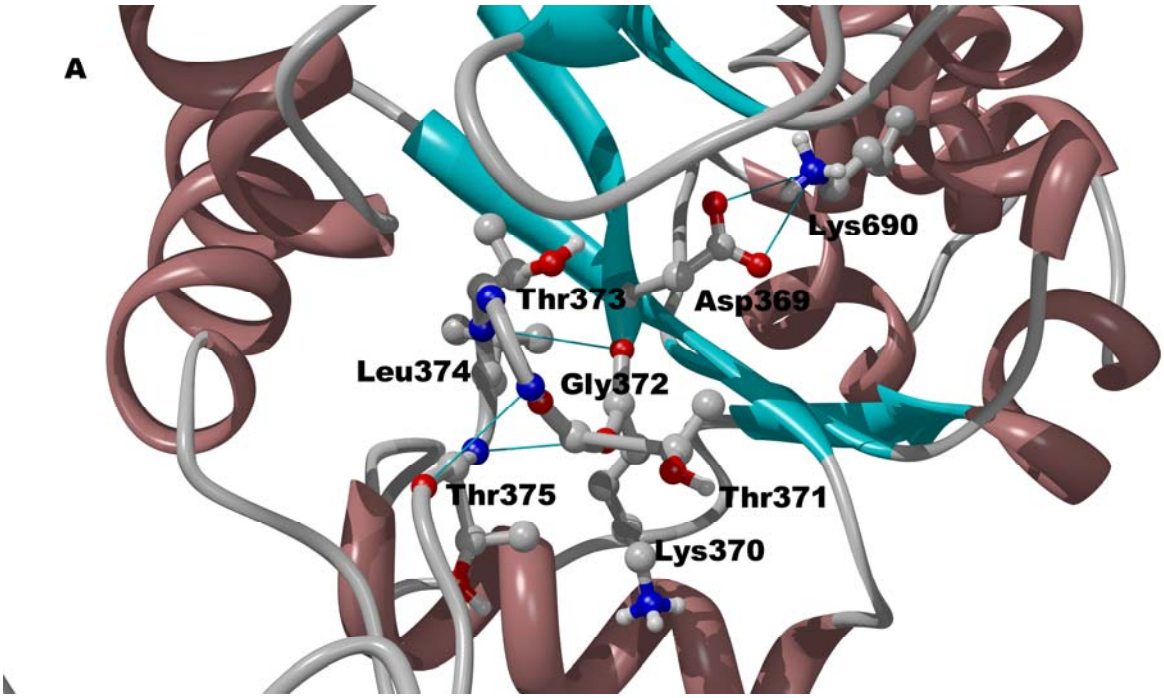

Figure 2B

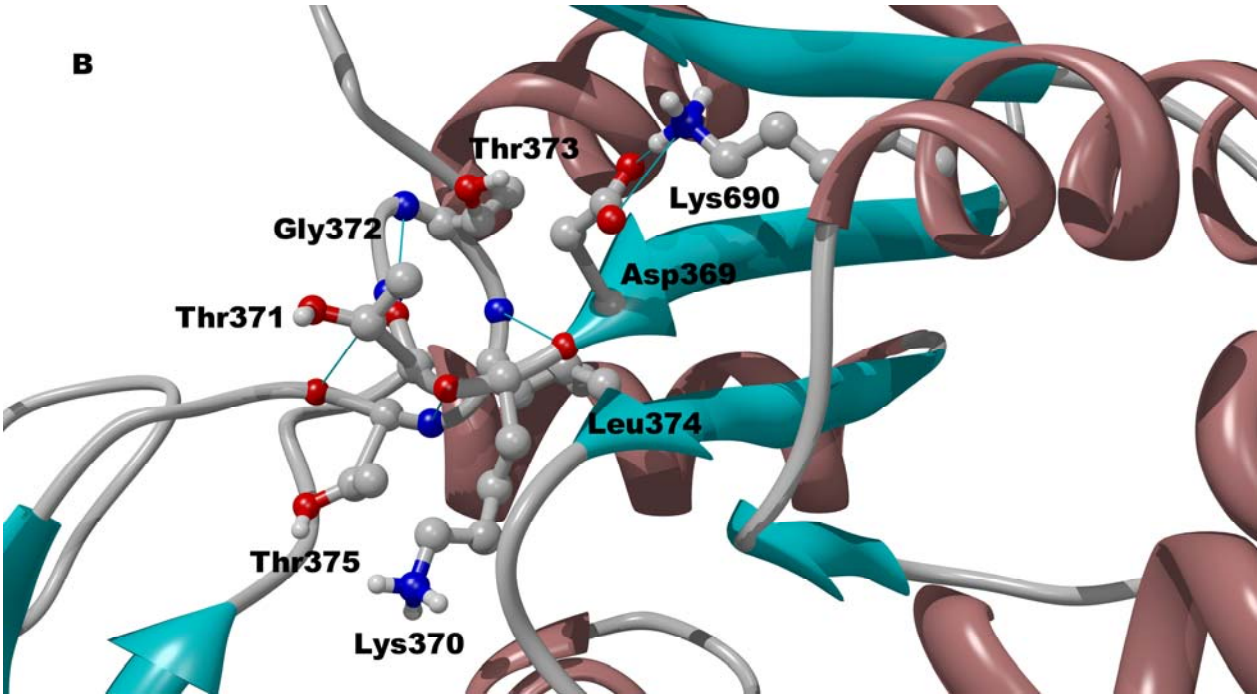

Figure 3A

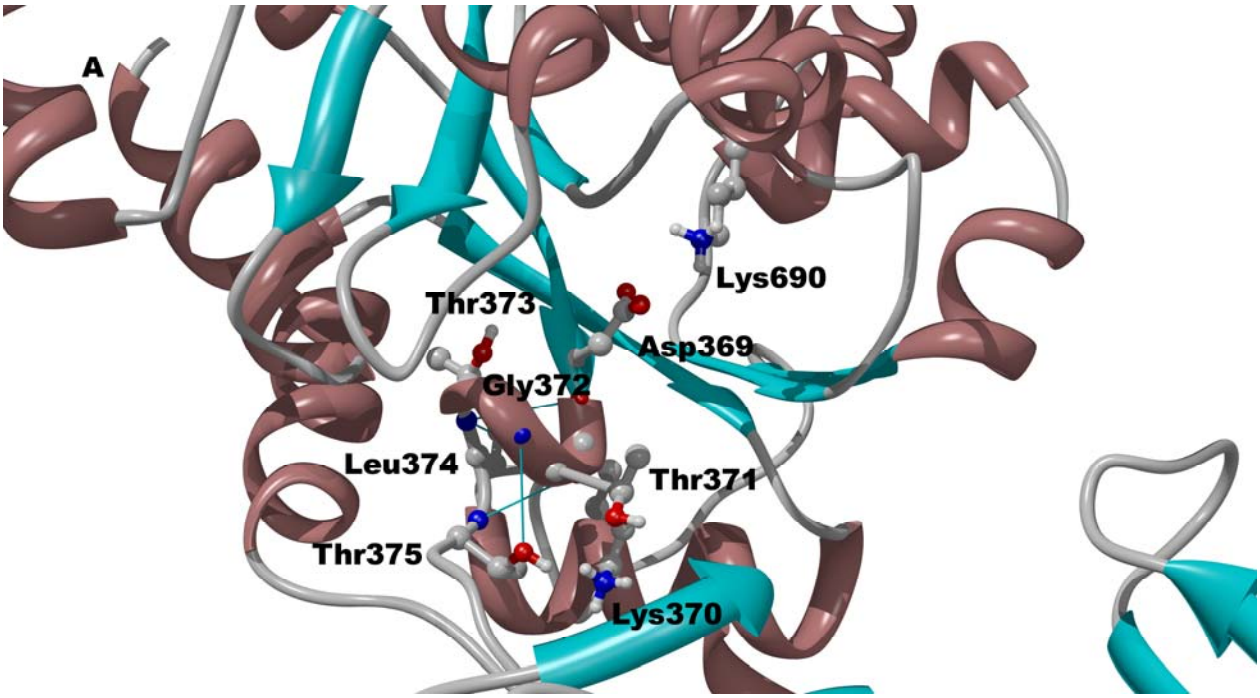

Figure 3B