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Computer modelling of human α_1-antitrypsin reactive site loop behaviour under mild conditions*

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Human α_1-antitrypsin (α_1-PI) is a member of the serpin superfamily of proteins. The reactive site loop (RSL) of the serpin binds to the active site of its target proteinase. Deficiency of α_1-antitrypsin is associated with a spontaneous conformational transition in the molecule which leads to a polymer formation. Mild conditions (1 M guanidinium·HCl), temperature and point mutations within the RSL are the factors that induce polymerisation. Initiation of this process has been associated with the disruption of a salt bridge Glu342→Lys290. In this paper the interaction of guanidinium ion with Glu342 and Lys290 as well as the effect of this interaction on the mobility of RSL is studied by molecular modelling.

Human α_1-antitrypsin (α_1-PI), the most abundant circulating proteinase inhibitor, is a member of the serpin superfamily of proteins [1]. The polypeptide chain structure of the inhibitor is highly ordered into three β-sheets (A, B and C) and several α-helices [2]. The region of α_1-antitrypsin that binds to the active site of its target proteinase (residues 343–363) is an exposed loop that has been called the reactive site loop (RSL). The RSL of small proteinase inhibitors, as those of the Kunitz family, are held in a tightly constrained conformation by interaction with the body of the inhibitor. The tertiary structure of the RSL (called the canonical form) is conserved among inhibitors from different families. The RSL conformation of small inhibitors is essentially the same in free and bound inhibitors, and it is thought that the stabilisation of its structure, by interaction with the body of the inhibitor molecule, is important for the formation of the tight complex with cognate proteinase [3]. In contrast to the tight constrained reactive site loop of Kunitz inhibitors, serpins have mobile RSL able to move in and out of the A β-sheet [4]. It is assumed that this mobility is critical to serpin function. The peptide bond between residues 358 and 359 of the antitrypsin RSL is cleaved if the inhibitor-proteinase complex is dissociated. The cleavage is followed by the insertion of 16 residues on the

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Abbreviations: ATIII, anti-thrombin III; GuHCl, guanidinium·HCl; MD, molecular dynamics; PAI-1, plasminogen activator inhibitor; α_1-PI, human α_1-antitrypsin; RAF, rotational autocorrelation function; RSL, reactive site loop.

amino terminal side of the RSL into the middle of the A sheet. This places residues 358 and 359 at opposite ends of the molecule, about 70 Å apart, and results in loss of inhibitory activity. Recent reports have confirmed that the exposed RSL is able to adopt other conformations such as in PAI-1 where full insertion of the intact RSL into the A sheet has been crystallographically demonstrated [5]. This conformation has been known as a latent state and is predicted for antitrypsin and found for ATIII [6]. The exposed, mobile RSL can also interact with the A and the C sheet of adjacent molecules leading to the polymerisation *via* A sheet and C sheet mechanisms, respectively. Mild conditions (1 M GuHCl), temperature and point mutations within the RSL are the factors inducing polymerisation. Our recent results suggest that GuHCl highly increases the RSL mobility [7]. This can be due to disruption of a salt bridge between Glu342 and Lys290. The lost salt bridge may cause insertion of the RSL into the A sheet with simultaneous release of 1C strand. The RSL of an adjacent molecule could thus insert into the gap created in the C sheet. In this paper we use molecular modelling to study the details of the interaction between guanidinium ion and the residues which comprise the salt bridge, as well as the influence of the ligand on the RSL mobility.

MATERIALS AND METHODS

The three-dimensional crystal structure of the cleaved α_1-antitrypsin molecule (Met358 and Ser359 of the reactive site loop are separated by approx. 70 Å) is deposited with the Protein Data Bank (7api) [2]. The structure was the initial configuration in the studies described in this paper. The crystal structure of the uncleaved α_1-antitrypsin molecule has been determined by X-ray crystallographic methods [8], however, it has not been deposited with Protein Data Bank yet, neither has it been made available to us otherwise.

All simulations described in this paper were performed with the molecular mechanical simulation package Amber 4.0 [9]. The united-atom OPLS [10] force field for the protein and ligand molecules and TIP3P [11] force field for the water were used. In the protein all terminal residues were charged and histidines were in the protonated form. In simulations where water was not included explicitly, to mimic the presence of water, the distance dependent dielectric constant was used, otherwise it was set to 1.0. A residue based cutoff radius of 12 Å was applied. The pair list was updated every 50 steps. The integration time step was set to 2 fs. The bonds involving hydrogen atoms were kept rigid during molecular dynamics simulations by using the SHAKE method [12].

Modifications of the protein structure were done with modules of BioSym program [13].

Our first goal was to construct a stable uncleaved form of α_1-antitrypsin closest to the one published by Song *et al.* [8]. The procedure to obtain it consisted of several steps. First, 4A strand and part of 1C strand, which comprise the reactive site loop in the native molecule (residues 343–363), were separated from the molecule. The two strands were linked into one fragment. Several torsion angles in the polypeptide chain of the fragment were changed to form a distorted helical conformation resembling the one found by Song *et al.* [8]. Three other conformations of the fragment were generated in a similar way. Each of the above four fragments (reactive site loops) was built into a modified protein molecule and four such generated protein structures were optimised by energy minimisation. In further studies the molecule of the lowest energy conformation was used.

Molecular dynamics (MD) simulation of the whole optimised structure of the uncleaved molecule was performed for 500 ps. During the first 150 ps temperature of the system was increased from 10 K to 300 K; the last 350 ps simulation was carried out at 300 K.

The first attempt to establish whether a guanidinium ion can change the stability of the salt bridge between Glu342 and Lys290 was to perform energy minimisation for the protein with the ligand for six different, arbitrary chosen starting arrangements. In four cases, the optimised uncleaved protein molecule was used; the ligand was placed in such a way that the distance between its closest nitrogen atom and the closest oxygen atom of the side chain of Glu342 was 2.70, 3.54, 3.97 and 4.47 Å, respectively. In one of the minimisations (3.97 Å) explicit water was included. In two other cases, the molecule conformation was the one obtained after 170 ps of MD simulation (cf. above)

and the distance between Glu342 and the ligand was 2.74 Å. One of the minimisations was performed in the absence and the other in the presence of water. In all calculations where water was explicitly included, only a fragment of the molecule comprising the reactive site loop and adjacent residues (residues 341–364 and 290) was hydrated with a spherical cap of Monte Carlo water. The number of hydrating water molecules was 605.

Finally, MD simulation was performed for the hydrated (see above) α_1-antitrypsin in the presence of 20 ligand molecules. The starting conformation of the protein was its dynamic structure obtained in the MD simulation without explicit water and ligand molecules, after 500 ps. The ligand molecules in the cationic form were randomly placed around Glu342 inside the water cap; their number corresponded to the concentration (1 M GuHCl) used in experiments [7]. No anions were added to neutralise the solution. In this simulation only the atoms of the hydrated residues and of the ligand and water molecules were allowed to move. The system temperature was set to 310 K.

RESULTS

The first step in the study of the effect of guanidinium ions on the behaviour of the reactive site loop of α_1-antitrypsin was the construction of an uncleaved molecule structurally similar to its active form [8]. The presumption that the guanidinium ion disrupts the salt bridge Glu342→Lys290 (Fig. 1) was tested in the series of energy minimisations of the protein with the ligand. To see the direct effect of the ligand on the mobility of the reactive site loop two MD simulations were performed: one of the sole α_1-antitrypsin molecule and the other of the molecule in the presence of ligand molecules and explicit water.

Optimised structure of the uncleaved α_1-antitrypsin

Four starting structures (cf. Materials and Methods) of the uncleaved form of α_1-antitrypsin obtained by model building were optimised by energy minimisation. The minimum energies for the four structures were –3940.5, –4000.3, –4001.8, –4026.4 kcal/mol, respectively. The structure with the lowest energy was used for further analyses. This structure resembles closely the structure of the uncleaved α_1-antitrypsin determined by Song *et al.* [8].

Molecular dynamics simulation of α_1-antitrypsin

Protein without ligands – system equilibration

MD simulation was carried out for 500 ps starting from the lowest energy, optimised structure of α_1-antitrypsin. The energy and temperature profiles of the system are shown in Fig. 2A. The potential energy stabilises after approx. 250 ps. For further analyses 200 ps trajectory (300–500 ps) was used.

Hydrated protein with ligands – system equilibration

MD simulation was carried out for 300 ps. The starting conformation of the protein was the dynamic structure obtained in the sole molecule MD simulation (see above) after 500 ps. Residues 341–364 and 290 were hydrated and 20 ligand molecules were placed around Glu342 inside the water cap. The energy and temperature profiles of the system are shown in Fig. 2B. The potential energy stabilises within 100 ps. For further analyses 200 ps trajectory (100–300 ps) was used.

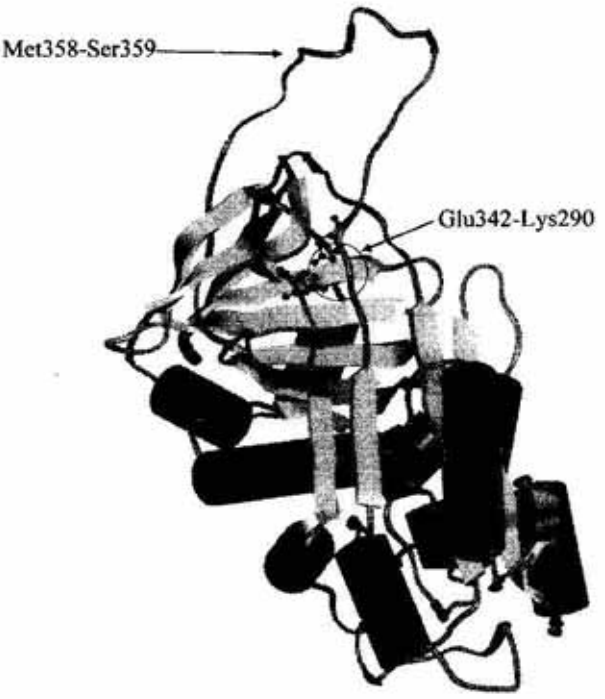

Fig. 1. The optimised structure of the uncleaved α_1-antitrypsin.
The place where the reactive site loop is cleaved after the inhibitor–proteinase complex is dissociated is shown. The salt bridge between Glu342 and Lys290 is also indicated.

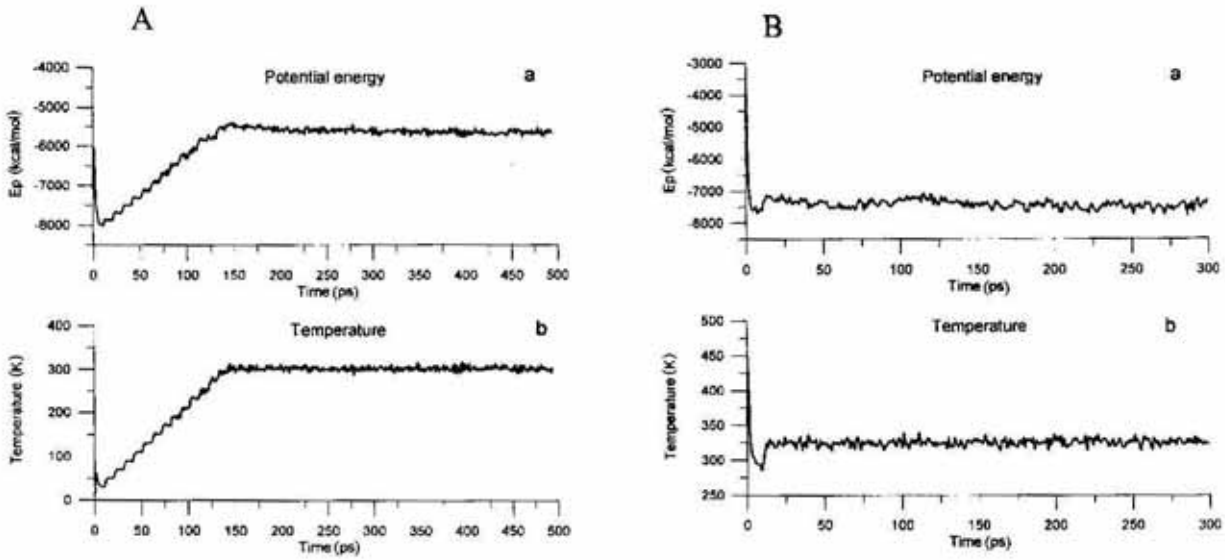

Fig. 2. The profiles of (a) potential energy and (b) temperature for the whole course of molecular dynamics simulation of α_1-antitrypsin without ligands (A); with ligands (B).

Effect of the guanidinium ion on the stability of the salt bridge Glu342→Lys290

Energy minimisation of α_1-antitrypsin with ligand

Six energy minimisations of α_1-antitrypsin with one ligand molecule were performed (cf. Materials and Methods).

In all cases, except for 4.47 Å, the final distance between the closest nitrogen atom of the ligand and the closest carboxylic oxygen atom of the side chain of Glu342 in the energy optimised configuration was approx. 3 Å. Both in the hydrated and non-hydrated systems, the distance between the carboxylic oxygen atom of Glu342 and the amine nitrogen atom of Lys290 slightly increased (0.03–0.20 Å) in the presence of ligand. Larger increase was observed in the cases when the initial structure was the one after 170 ps of MD simulation. Such results suggest that guanidinium ion might destabilise Glu342-Lys290 interaction.

Molecular dynamics simulation of α_1-antitrypsin without ligand

To see a direct influence of the guanidinium ion on the stability of the salt bridge Glu342→Lys290 MD simulations of the protein in the absence and in the presence of ligand molecules were performed. In the absence of ligand the distance between the atoms forming the salt bridge (i.e. an oxygen atom of the Glu342 carboxyl group, OE1, and the nitrogen atom of the Lys290 amine group, NZ) is approximately constant over the whole course of simulation (Fig. 3a). However, in the dynamic structure of the protein the distance is smaller by about 1.0 Å than that in the crystal (Fig. 3a). Geometrical analysis of the relative arrangement of OE1 and NZ in the dynamic structure of α_1-antitrypsin indicates the formation of a tight hydrogen bond (H-bond) between Glu342 and Lys290, whereas in the crystal (7api) such a H-bond is not formed. Thus, the interaction between these two residues is weaker in the crystal.

Molecular dynamics simulation of hydrated α_1-antitrypsin with ligand

The disruption of the salt bridge Glu342→Lys290 induced by the presence of a guanidinium ion resulted from the formation of stable H-bonds between an oxygen atom of the Glu-342 carboxyl group, OE1, and a nitrogen atoms of the ligand molecule, and also between the nitrogen atom of the main chain of Lys290 and the oxygen atom of Pro219. A H-bond was considered stable if its life time was longer than 100 ps.

In MD simulation one of the ligand molecules forms stable H-bonds with Glu342 within first picoseconds. In this interaction, the carboxylic oxygen OE1 of the side chain of Glu342 forms, for most of the time, H-bonds with two nitrogen atoms of one ligand molecule (Figs. 3 and 4). After about 230 ps, another stable H-bond is formed between another ligand molecule and the second carboxylic oxygen atom, OE2, of Glu342. Concomitant with the forma-

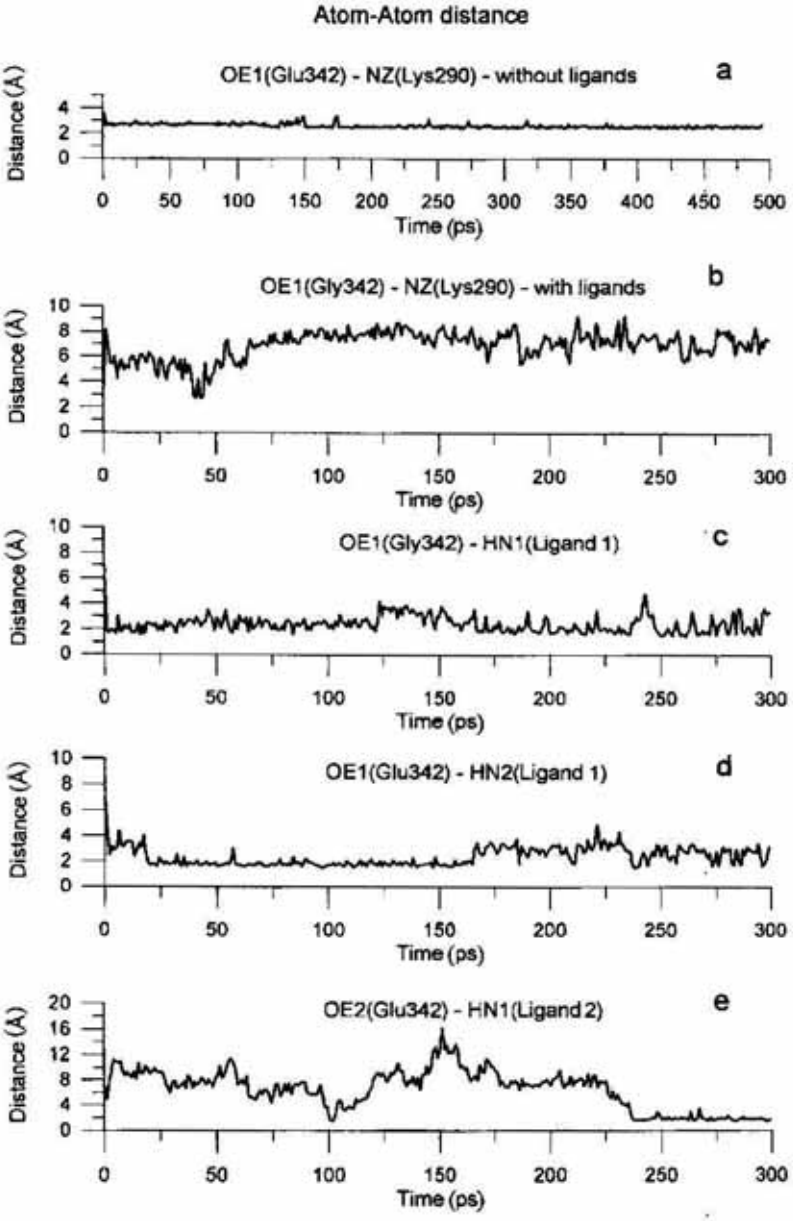

Fig. 3. The history of the salt bridge in molecular dynamics simulations of α_1-antitrypsin without and with ligands.
The distance between the atoms comprising the salt bridge (OE1 of Gly342 and NZ of Lys290) in simulation (a) without ligands and (b) with ligands. The distance between OE1 (one of the carboxylic oxygen atoms of Glu342) and two hydrogen atoms, HN1 (c) and HN2 (d) of one ligand molecule, Ligand 1. The distance between OE2 (the other carboxylic oxygen atom of Glu342) and a hydrogen atom, HN1 (e) of another ligand molecule, Ligand 2.

tion of the first H-bond between Glu342 and a ligand molecule, the side chain of Lys290 moves away from Glu342 and its main chain nitrogen atom eventually forms a H-bond with the main chain oxygen atom of Pro219. During the first picoseconds, when the first hydrogen bond between OE1 and ligand is formed, there is no water molecule in the vicinity of Glu342. However, in later times, short lived (less than 40 ps) H-bonds between OE1 or OE2 and water are occasionally formed (OE1 and OE2 have the same point charges).

Figure 3 shows relative distances of the atoms in Glu342 and Lys290 engaged in the salt bridge during the course of simulation (a) without ligand, (b) with ligands, as well as the relative distances of atoms in Glu342 and ligand molecules engaged in H-bonding (Figs. 3c, d, e). In the absence of ligand molecules (simulation started from the optimised crystal structure) the initial distance between OE1 of Glu342 and NZ of Lys290 is about 3.5 Å. Such a distance does not indicate a formation of a H-bond. Within the first picoseconds, the distance decreases to an average value of 2.55 Å ± 0.08 indicating a formation of a strong H-bond (the average value of O---N—H angle is 7.6° ± 4.18). In the presence of ligand molecules the initial distance between OE1 and NZ is about 2.6 Å (simulation started from the dynamic structure). Within the first picoseconds, the distance increases to an average value of 7.0 Å ± 1.2. At the same time, a ligand molecule, initially away from Glu342, approaches it and a stable H-

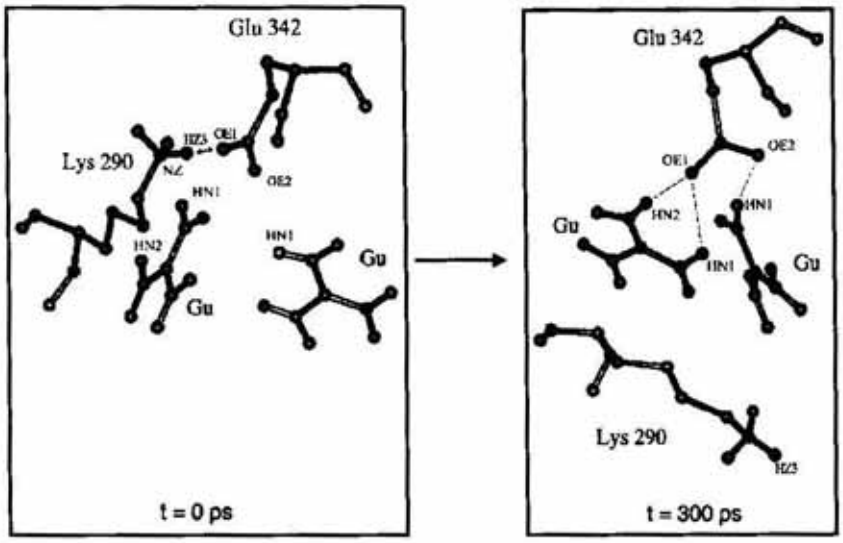

Fig. 4. Two snapshots of the relative arrangement of Glu342, Lys290 and two ligand molecules, Gu, at the initial time (t = 0 ps) and after 300 ps molecular dynamics simulation (t = 300 ps).

bond is formed between OE1 and one of the nitrogen atoms of the ligand (Fig. 3c). After a few picoseconds a second H-bond is formed between the same oxygen atom and another nitrogen atom of the same ligand molecule (Fig. 3d). Another ligand molecule makes the third H-bond with Glu342 after about 230 ps (Fig. 3e). In this bond the second carboxylic oxygen, OE2, of the Glu342 side chain is engaged (Fig. 4).

Mobility of the α_1-antitrypsin RSL

Stability of the reactive site loop was described in terms of the formation of H-bonds between atoms of the site loop residues (343–363) and atoms of other residues of α_1-antitrypsin. Also, by calculating rotational auto- correlation function of C—N bond of Gly349 which is the central residue of the reactive site loop. Results for the molecule in the absence of ligands were compared with those for the molecule in the presence of ligands. As above, a H-bond was considered stable if its life time was longer than 100 ps.

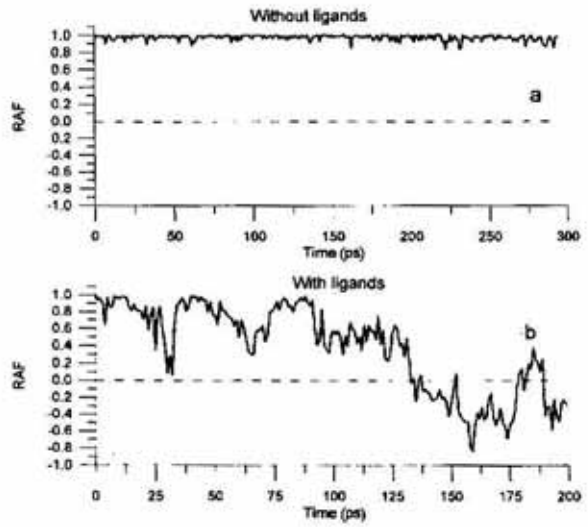

Fig. 5. Rotational autocorrelation function of the C–N bond of Gly349 (centre of the reactive site loop) for molecular dynamics simulation of α_1-antitrypsin (a) without ligands and (b) with ligands.

H-bonding within α_1-antitrypsin without ligand

One of the carboxylic oxygen atoms of Glu346 forms stable H-bonds with both the nitrogen atom in the side chain of Arg196 and the nitrogen atom of the side chain of Lys201. The other carboxylic oxygen atoms of Glu346 form stable H-bonds with two nitrogen atoms of the side chain of Arg196. Also, the oxygen atom of the main chain of Ile356 makes two H-bonds with the oxygen atom of the hydroxyl group of Ser285 and a nitrogen atom of the ring of His287. The carboxylic oxygen of Glu363 forms a stable H-bond with the nitrogen atom of the amine group of Lys368. Much less stable H-bonds are made between Glu354 and Ser359 within the reactive site loop. Although temporary H-bonds involving sulphur atoms are formed, their lifetimes are too short for them to contribute significantly to the stability of the reactive site loop structure.

H-bonding within α_1-antitrypsin with ligands

Some of the intramolecular H-bonds present in the system without ligand molecules are found in the system with ligand molecules: Glu346 forms H-bonds with Arg196 and Lys201 as well as Glu363 forms H-bond with Lys368. However, the H-bonds made by Ile356 with

Ser285 and His287 appear only initially. In their place a H-bond between the oxygen atom of the main chain of Glu363 and the oxygen atom of the side chain of Ser285 is formed. The "intra-loop" H-bond between Glu354 and Ser359 is not formed.

In the presence of ligands, the reactive site loop assumes rather an upward whereas in the absence of ligands rather a leaning towards the molecule position.

Rotational mobility of the reactive site loop of α_1-antitrypsin without ligand

Rotational autocorrelation function (RAF) of C—N bond of Gly349 in the centre of the reactive site loop was calculated (Fig. 5a). The function practically remains constant and equal to 1.0 for 300 ps analysis time. Small noise present in the function indicates that the bond wobbles inside a very restricted volume.

Rotational mobility of the reactive site loop of α_1-antitrypsin with ligands

Rotational autocorrelation function of the same bond in the presence of ligands was calculated for 200 ps analysis time (Fig. 5b). The function decays to zero within approx. 130 ps and then becomes negative. Large noise present in the function indicates high amplitude wobbling of the bond. The change in sign of RAF means that the bond changes its direction with time — this can result only from a substantial bending of the loop.

DISCUSSION

It is now widely accepted that deficiency of α_1-antitrypsin is associated with a spontaneous conformational transition in the molecule which leads to a polymer formation. The mutation in Z α_1-antitrypsin involves replacement of only one amino acid at the base of the reactive site loop (RSL) of the molecule (glutamic acid at position 342 is replaced by lysine) [14]. This replacement is responsible for the loss of a salt bridge occurring in the intact inhibitor between Glu342 and Lys290. This leads to a high increase in the RSL mobility allowing spontaneous polymerisation of the variant by the insertion of its RSL into the A sheet of an adjacent molecule.

The formation of α_1-antitrypsin polymers is observed in the presence of 1 M GuHCl [7] at physiological temperature. It has been postulated that breaking of the salt bridge is also involved in the process of native inhibitor polymerisation [7]. The above presumption, based on the experimental results, was tested in the course of molecular dynamics simulations. In the systems containing the inhibitor and guanidinium ions (their concentration corresponded to 1 M solution) there are two concomitant processes initiated within the first picoseconds. The first, of forming stable H-bonds between one of the ligand molecules and Glu342. After about 200 ps another ligand molecule makes a H-bond with the same residue. The second, of moving Lys290 away from Glu342. Lys290 eventually forms a stable H-bond with Pro219. In the absence of ligands the distance between Glu342 and Lys290 decreases from its initial value of 3.5 Å in the crystal to 2.55 Å. The mobility of the RSL of α_1-antitrypsin in the presence of ligands is much higher then that in the absence of ligands.

A strong H-bond between Glu342 and Lys290 in the dynamic form of α_1-antitrypsin without ligand would indicate that stabilisation of RSL, due to Glu342-Lys290 interaction, might be higher than it was anticipated from the salt bridge distance in the crystal form. Formation of the H-bond could explain the results of Mast *et al.* [15] and Lomas *et al.* [16] that polymerisation for native inhibitor takes place at high temperature compared to that inducing polymerisation for α_1-antitrypsin with Z mutation which lacks Glu342-Lys290 interaction.

Experimental results suggest that the GuHCl induced disruption of the Glu342→Lys290 salt bridge increases the RSL mobility and enables insertion of the RSL into the A sheet at the depth of at least five residues [7, 17]. The insertion of α_1-antitrypsin RSL into its own A sheet might be accompanied by the release of 1C strand which creates a free space for binding the RSL of an adjacent molecule. MD simulation shows that in the presence of ligands a new stable hydrogen bond is formed between Glu363 (which belongs to 1C strand) and Ser285. This might indicate that the 1C strand is pulled out of the C sheet in these conditions. If this mechanism were proven in further computer modelling studies it would explain the C sheet polymerisation observed experimentally in the presence of GuHCl [7].

Although in MD simulation of the protein without ligands water was not explicitly included, the effect of water was approximated by applying a distance dependent dielectric constant. Such an approximation adequately models the screening effect of water, however, it cannot model direct interaction between the molecule and solvent. For this reason, a special attention was paid to establishing whether in the explicitly hydrated system which includes ligands, direct H-bonds with water in the site of Glu342 and Lys290 are formed. During the first picoseconds of simulation, when the disruption of the salt bridge occurs, such bonds have not been formed. This result strongly suggests that the disruption of the salt bridge in mild conditions is due entirely (mainly) to the interaction of guanidinium ions with Glu342. Although direct effect of water or of the net positive charge of the hydration sphere cannot be totally ruled out at this stage, their significant contribution to the salt bridge disruption would be at variance with experimental results [17]. Nevertheless, we will continue this study to get better understanding of the basic processes leading to α_1-antitrypsin polymerisation.

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