Study of 3D Structural Model of Granzyme H to Predict Different Sites Involved in Substrate and Inhibitor Recognition during Apoptosis

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Abstract

Apoptotic cell death is known to regulate the maintenance of the normal physiological state and the pathogenesis of diseases. Virus infected and tumor cells are got rid of by natural immune system of the body using granule exocytosis (T lymphocytes and natural killer cells). In the present study, 3D structural model of enzyme was used to determine the different site of granzyme H involved in substrate and inhibitor recognition during apoptosis. It has been found from the results presented here that certain amino acid in the structure of granzyme H may function as recognition site for the substrate binding and consequently mutation in these amino acid sequences could affect the normal process of catalysis.

Keywords: Autolysis, Granzyme H, Mutation, Apoptosis.

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INTRODUCTION

Apoptotic cell death plays an important role in the maintenance of the normal physiological state and in the pathogenesis of diseases (Loro etal., 2005). Granule exocytosis is the main pathway for the immune elimination of virus-infected cells and tumour cells by cytotoxic T lymphocytes and natural killer cells (Chowdhury and Lieberman, 2008). Natural killer (NK) and cytotoxic T-lymphocytes (CTLs) destroy cells within an organism to defend it against viral invasion and tumor growth. They kill target cells by multiple mechanisms. Upon interaction with target cells, cytotoxic T lymphocytes and natural killer cells vectorially secrete highly specialized cytoplasmic granules containing perforin and a family of 11 serine proteases or granzymes (Smyth and Trapani, 1995) . The exocytosis of death-inducing granzymes stored in the granules of cytotoxic lymphocytes allows the immune system to rapidly eliminate intracellular pathogens and transformed cells. The membrane-disrupting protein perforin allows the entry of granzymes into the target cell where they induce apoptosis by cleaving target substrates in the cytoplasm and nucleus (Ashton-Rickardt, 2005). Granzymes are synthesized as zymogen precursors containing signal and a short pro-peptide preceding the mature polypeptide chain . Transport of zymogens through the endoplasmic reticulum and Golgi network before recognition by cathepsin C requires a certain degree of conformational stability, which prevents both premature proteolytic activity and degradation of

precursors intra-cellularly (Wiltrout et al., 1996). The removal of N-terminal pro dipeptide is ensured by lysosomal cysteine protease cathepsin C (Hink-Schauer et al., 2002). Granzyme family is divided into subfamilies, viz, tryptase, chymotrypsin-like protease and metase. Granzymes have features that are strongly conserved including: consensus amino acid sequences at their N-termini and around the three catalytic residues, activation from zymogenic forms, and conserved disulphide bridges (Toomes et al., 1999).

Granzymes kill cells in a variety of ways. The work has demonstrated that granzymes induce mitochondrial dysfunction through caspase and caspase-independent pathways and induce damage to DNA and the integrity of the nucleus (Smyth et al., 1996). Caspase independent pathway demonstrated that perforin and serine proteinases synergistically trigger an endogenous pathway of apoptosis resulting in dissolution of the target cell nuclear membrane and DNA fragmentation. Neither perforin nor granzymes possess inherent nuclease activity, but in combination they can induce target cell apoptosis. It is hypothesized that perforin enables effectors granzymes to enter the target cell cytoplasm and following their transport into the nucleus, granzymes cleave specific target cell nuclear proteins to activate autolytic endonucleases that fragment DNA. Recently, it is reported that the granzymes have two cationic sites; cs1 (surface loop) and cs2 (heparin binding motif). These binding sites participate in the binding of granzymes to cell surface, thereby promoting its uptake and release from the cytotoxic lymphocytes to the cell cytoplasm of virus or tumor or cell that undergo autolysis (Smyth et al.,1994). In the cell it causes the cleavage of proteins at its specific site like tyrosine or phenylalanine thus shows chymotrypsin like activity. This cleavage stimulate the process of proteolysis which may cause the mitochondrial disruption (caspase independent pathway) (Bird et al., 2005), or may stimulate the conversion of procaspase to caspase which acts on the nuclear protein like polyamino ribose polymerase and causes DNA fragmentation that leads to cell death (caspase dependent pathway) (Harris et al., 1998, Jans et al., 1999).

In addition to granzyme A and B, a third member of this family has been cloned and designated granzyme H (a natural serine protease). It is found that granzyme H is more abundant in Natural Killer (NK) cells than other granzymes (Fellows et al., 2007). The gene that encodes this granzyme is located between the granzyme B and cathepsin G genes on human chromosome 14q 11.2. (Godfrey and Trapani 2004).The granzyme H shows the highest degree (greater than 54%) of amino acid sequence homology with granzyme B and cathepsin G and, like these genes, consists of five exons (Maclovor et al., 1999). Their generally accepted mode of action consists of their directed secretion towards a virus-infected or neoplastic target cell and perforin-dependent delivery to the target cell cytosol, where they engage in various actions resulting in target cell apoptosis (Haddad et al., 1991). It is observed that like granzyme B, granzyme H also has chymotrypsin like activity (Regner and Mullbacher, 2004).

We have previously predicted the structure of granzyme H for the first time (Edwards et al., 1999). It was observed that granzyme is composed of 4 alpha helices and 9 beta strands with an overall a/b fold common for globular protein. N-terminal isoleucine and C-terminal lysine is stabilized by H bonding. Stability of granzyme is enhanced by entropic effect of 3 disulfide bridges. The catalytic site of granzyme H comprises side chains of Ser202, His64 and Asp108.

We present in this study the mutational aspects of granzyme H. The structural studies would serve as an insight towards understanding the role of this tumor marker specifically its role in the process of apoptosis that may help in cancer metastasis.

MATERIALS AND METHODS

Sequence Retrieval and Comparison

Amino acid sequence of granzyme H was retrieved from SWISS PROT databank (Khurshid etal., 2001). The sequence was submitted for search against Protein databank, PDB (Bairoch and Apweiler 1997), using Gapped BLAST and PSI-BLAST algorithm (Berman et al., 2000). The

protein that gave the highest homology (95%) to yield a meaningful sequence alignment with a large fragment of granzyme H (21-244) was granzyme B (Regner and Mullbacher 2004). This fragment matched with the residues 21-244 of granzyme.

Secondary Structure Prediction

The secondary structure prediction of granzyme H was carried out using the PHD method (Altschul et al., 1997), by submitting the sequence to Predict Protein Server at the European Molecular Biology Laboratory (EMBL).

Secondary Structure Prediction

Comparisons in the sequence of granzyme H were done with other members of the granzyme family i.e. granzyme A, C, D, E, M and K. The multiple sequence alignment was generated by Clustal W program (Rost and Sander 1993)

Protein Pattern Search

Primary sequence of granzyme H was searched at PROSITE database (Thompson et al., 1994). PROSITE (a dictionary of protein sites and patterns) is a method of determining the function of uncharacterized proteins translated from genomic or cDNA sequences. It consists of a database of biologically significant sites and patterns formulated in such a way that with appropriate computational tools it can rapidly and reliably identify to which known family of protein the new sequence belongs.

Model building and Evaluation

Three dimensional model of granzyme H was constructed using the crystal coordinates of granzyme B. The automated homology model building was performed using the protein structure-modeling program MODELLER 9. Each model was selected after many runs of the MODELLER (more than 10) in order to achieve the most plausible model satisfying many stereochemical criteria. At each step, the models built by the MODELLER were checked by PROCHECK (Bairoch and Apweiler 1997) and the final model was chosen that fits or nearly fits the criteria of an accepted model. In addition, the variability among the models was compared by superposition of Cα traces and backbones onto the template crystal structure from which the r.m.s. value for positional differences between equivalent atoms could be calculated. The structural super positions were performed by using options available in the SUPERPOSE command of MODELLER.

Model Analyses

Mutational studies and calculations for ion pairs, accessibilities and hydrogen bonds for each model were done by Vriend 1990.

(a) Mutational Studies

Mutational Studies were performed by the MUTATE command within the WHAT IF package.

(b) Ion pairs

The tight ion pairs in proteins have been postulated to have interatomic distances £ 4.0Å by (Vriend 1990). The analysis was performed by SHOSBR (show salt bridges) command within the ANACON (Contact Analysis) menu in the WHAT IF package.

(c) Hydrogen Bonds

Hydrogen bond analysis was done by the HBONDS module of WHAT IF. WHAT IF uses four parameters to determine if a hydrogen bond can be formed between the hydrogen of the donor atom and the lone pair of the acceptor atom. The default parameters are:

(i) Maximal distance between the donor and the acceptor atoms = 3.5Å

(ii) Maximal distance between the calculated hydrogen position and the acceptor atom = 2.5Å

(iii) Angle from the donor atom over the hydrogen to the acceptor atom = 60^0

(iv) Angle from hydrogen over the acceptor to the atom to which it is covalently attached = 90^0

Separate evaluations of backbone-backbone (BB), backbone-sidechain (BS) and sidechain-sidechain (SS) hydrogen bonds were also done by the BSSHBO command within the HBONDS module. The statistics adopted by WHAT IF are those described by Baker and Hubbard (1984).

(d) Solvent accessibility

The accessibility calculations were done by the ACCESS (surface area calculations) menu of WHAT IF.

RESULTS AND DISCUSSION

General model description

The predicted 3D homology models shows a conserved two similar domain structure, i.e., an N-terminal domain and a C-terminal domain comprising predominantly of beta-sheet structure with a little alpha-helical content. As in other chymotrypsin-like serine proteases, the single GzmH polypeptide chain folds into two β -barrels, each comprising six antiparallel strands labeled β1 to β 6, and β 7 to β 12. Two helical elements are found on the surface of the molecule, a single 3_{10} turn (Cys170 to Leu175, α1) and a long C-terminal α -helix extending from Pro237 to Asn243 (α2) (Figure 1).

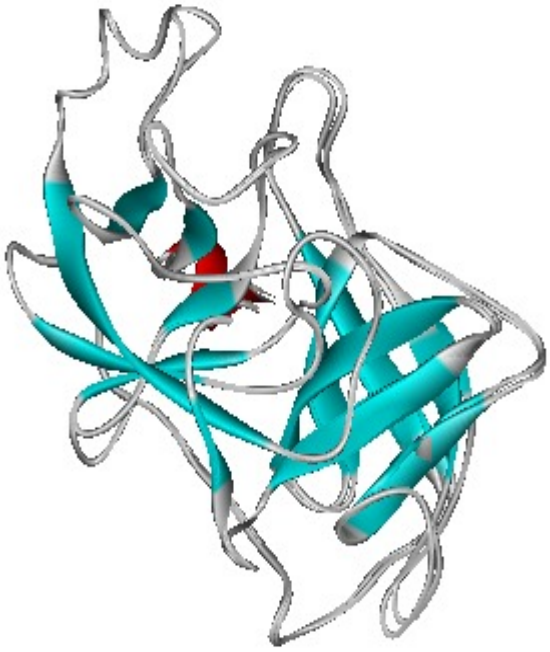

Figure 1: Structural superposition of beta traces of GzmB template and GzmH.
R.m.s. deviation = 2.5 A

Model Assessment

Table 1 shows the Ramachandran plot statistics of granzyme H obtained with PROCHECK (Bairoch and Apweiler 1997). The plot shows that 88.8% residues lie in the most favored region, 10.1% residues in the allowed region and 0.5% in the generously allowed region.
On the other hand, 0.5% residue lies in

Table 1: Procheck summary of the GzmH model.

```
+----------<<< P R O C H E C K   S U M M A R Y >>>----------+
|                                                            |
| 1lxv  2.5                                  224 residues |
|                                                            |
*| Ramachandran plot:  88.8% core  10.1% allow   .5% gener   .5% disall |
|                                                            |
*| Gly & Pro Ramach:    2 labelled residues (out of 34)              |
+| Chi1-chi2 plots:     2 labelled residues (out of 132)             |
|                                                            |
| Main-chain params:   6 better   0 inside   0 worse             |
| Side-chain params:   5 better   0 inside   0 worse             |
|                                                            |
*| Residue properties: Max.deviation:   4.6          Bad contacts:   2 |
*|              Bond len/angle:   6.0   Morris et al class:  1  1  2 |
+|   2 cis-peptides                                           |
| G-factors        Dihedrals:  -.04 Covalent:  -.22   Overall:  -.10 |
|                                                            |
| M/c bond lengths: 89.3% within limits 10.7% highlighted            |
| M/c bond angles:  80.0% within limits 20.0% highlighted            |
| Planar groups:  100.0% within limits   .0% highlighted             |
|                                                            |
+------------------------------------------------------------------+
 + May be worth investigating further.  * Worth investigating further.
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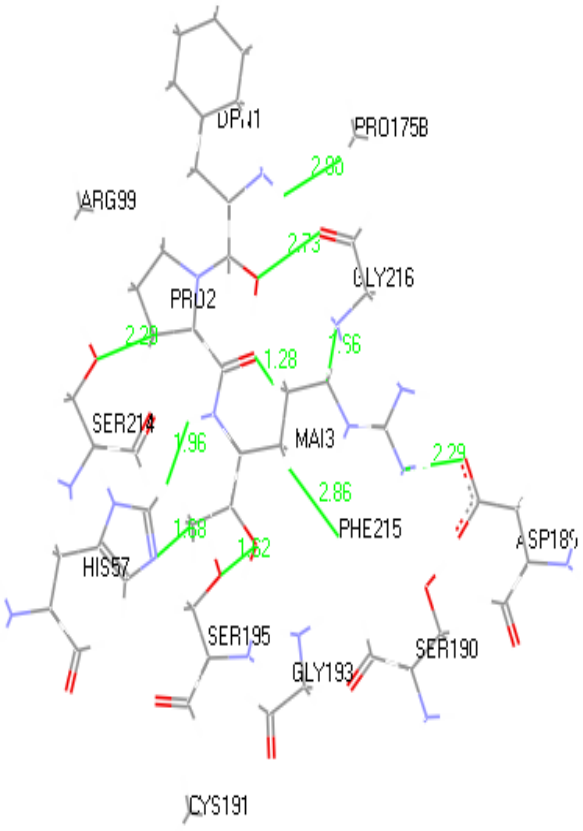
disallowed region of the plot. It was observed that the structurally conserved regions (SCR) in granzyme H (target) and the corresponding SCRs of granzyme B (template) display greatest structural similarity (71%). The root mean square (rms) deviations after superposition were calculated as 0.1924. (Figure 2)



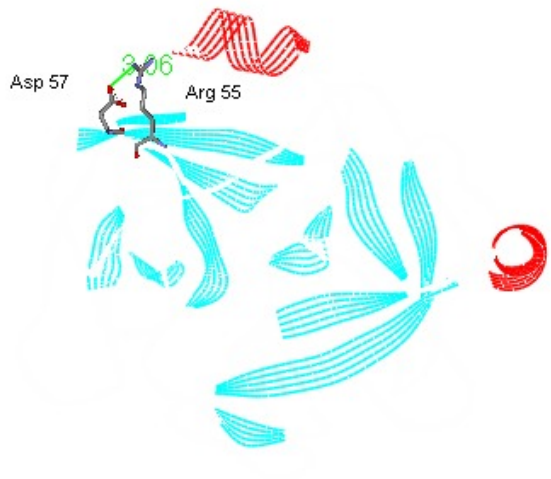


Fig: H-bond between Arg55 and Asp57 (dist 3.06A)

Catalytic triad in granzymes

The catalytic residues of GzmH are His64, Asp108 and Ser202 located at the junction of the two -barrels. The preformed active-site cleft runs perpendicular to this junction along the surfaces of both barrels. This catalytic triad form H-bonding and salt bridges. It is predicted that in the homology model N and ND1 atoms of His64 form H-bond with OD1 and OD2 of Asp108 respectively. Asp108 in turn forms a H-bond with Ser217. The catalytic complex is further stabilized, when the OG atom of Ser202 also forms H-bond with O of Ser217. Four salt bridges between His64 and Asp108 were also observed (Table 2). In granzyme K the catalytic triad residues are Ser195, His57, and Asp102 which are located in the center of the active-site cleft as in functional active proteases (Wiltrout et al., 1996). On the other hand, in granzyme B the catalytic triad residues are His57, Ser195 and Asp102 (Baker EN and Hubbard 1984). The catalytic triad residues GzmM are His41, Asp86, and Ser182 (Estebanez-Perpina et al., 2000).

Table 2: Predicted H-bonding and salt bridges of catalytic triad in Granzyme H

H-bonding	Salt Bridges
His64 (N)---(OD1) Asp108	His64 (OD1)---(ND1) Asp108
His64 (ND1)---(OD2) Asp108	His64 (OD1)---(NE2) Asp108
Asp108(OD2)---(OG2) Ser217	Asp108(OD2)---(ND1)His64
Ser202(OG)---(O) Ser217	Asp108(OD2)---(ND1)His64

Predicted mechanism of action

It is reported that antiviral and antitumor immunity of granzymes depends on the recognition, engagement, and destruction of infected or malignant cells by cytotoxic lymphocytes (Maharus et al., 2004). Killing of abnormal cells by cytotoxic lymphocytes (CLs) involves activation of target cell death receptor pathways or the release of CL cytotoxins (granzymes) into the target cell cytoplasm. Mechanism of action of granzyme especially of granzyme B was proposed by a group of workers. It is proposed that GzmB is a cationic protein having two binding sites located on the opposite side of the molecule to the catalytic pocket and are part of a larger cationic surface. These binding sites (specific non catalytic region) were designated as cationic sequence (cs1) and cs2. Among these, cs2 is a positively charged motif in the protein that may adopt either α-helical or β-strand conformation (XBBXBX or XBBBXXBX, where B is a basic amino acid and X is uncharged or hydrophobic) bind with galactose amino glycan (GAG's) and help in entering of granzyme in cell cytoplasm. On the other hand cs1 motif is a surface loop containing an exact match to the XBBXBX motif (110RKAKRTRA), where the underlined characters represent the putative heparin-binding motif. This heparin binding (exo) site could contribute to the recognition of macromolecular substrates, as shown *e.g.* for -thrombin, resulting in a highly selective function *in* vivo (Wiltroutet al., 1996). These cationic sites play a role in uptake of granzyme, intracellular trafficking, or perforin-mediated release into the cytoplasm where granzymes shows cytotoxic function (Smyth et al., 1994). Present study also revealed these cationic sequence, cs1 & cs2. GzmH model exhibited a surface loop containing an exact match to the XBBXBX motif (116RKAKWTTA) the cs1 binding site. It is observed that it is a part of the amphipathic C-terminal helix that has paired basic residues nearby the N-terminal region of GzmH. In the motif of these basic residues Arg116 shows salt bridge with its neighbors Glu115 and Lys117. On the other hand the NZ atom of Lys117 shows ionic pairs with OD1 of Asp57 and OD2 of Asp108 (a

catalytic residue. The cs2 binding site of GzmH is like other granzymes is a positively charged motif in the protein that adopts β-strand conformation (XBBBXXBX, where B is a basic amino acid and X is uncharged or hydrophobic) having 192KKTQTGF. Among this cs2 motif the NZ atom of Lys192 form ion pairs with OD1 and OD2 of Asp190. Where as the NZ atom of Lys193 forms ion pair with OD2 of Asp190. The amino acid of cs2 binding sites in GzmH also shows a network of H-bonds with amino acids like Gly223 and Asp210. There is inter net work of H-bonding is also observed among the amino acid residues of cs2 binding sites (Table 3). This may confirm that the putative heparin-binding motif of cs1 and the cs2 have the ability to bind with GAGs with the help of its polar and catalytic amino acid residues. It is therefore possible that the cationic sites may participate in binding of GzmH to the cell surface, thereby promoting its uptake and eventual release into the cytoplasm, where GzmH shows antiviral and antitumor immunity that may complement the proapoptotic function of Gzm in human NK cells confirming the studies of Hou et al (2008).

Table 3: H-bonding and salt bridges of peptide.

H-bonding	Salt Bridges
Ala118 (N)---(O) Lys56	Lys117 (NZ)---(OD1) Asp57
Leu114 (N)---(O) Asp57	Lys117 (NZ)---(OD2) Asp108
	Glu115(OE1)---(NH2)Arg116
	Glu115(OE1)---(NH1)Arg116
	Glu115(OE2)---(NH1)Arg116

Substrate specificity of Granzyme H

It is predicted by out modeling study that Arg208 occupies the corresponding position in GzmB. This residue has been shown to be important for conferring the substrate specificity to the enzyme in that it is capable of electrostatically attracting the negatively charged acidic side chains of Asp or Glu (Harris et al., 1998). Our study contradicts this study and found that instead of Arg208, the amino acid residue Cys is present at the position of 208. It is also noted that like Arg208, it is not attracted with any charged amino acid residue. The residue of Cys208 forms 2 H-bond with non polar amino acid Val211. Cys208 form a disulfide bond with Cys142. Whereas the Cys142 form two H-bonds with non polar amino acid Leu164. It may be possible that the disulfide bond of Cys208-Cys142 and their H-bonding and hydrophobic interaction shows the substrate specificity. Our study may confirm the study of Harris et al (1998) who reported that the Gzms form stable disulfide linked homodimer and trigger cell death in a caspase independent manner. The study of Facchiano et al (2006) proposed that the Asp specific site is present in the apoptotic promoting caspase. They found that an ideal peptide Ile-Glu-Pro-Asp is specific for apoptotic activity. Present study also observed such site in GzmH i.e. a peptide of Leu114-Glu115-Arg116-Lys117-Ala118 is a segment of cationic site cs1. It is possible that this site may be an apoptotic promoting caspase in GzmH due to the H-bonding of Leu114 and Lys117 with Asp57. The Asp57 forms many H-bonds with Arg55 (Table 4). It is reported by Harris et al (1998 that Arg55 shows an apoptotic promoting caspase activity. Arg55 and Cys49-Cys65 disulfide bond may also shows an apoptotic promoting caspase activity. Arg55 forms a number of H-bonding interactions and salt bridges with the other amino acids (Table 4). The presence of Arg55 near such an important site and its elaborate H-bonding network with other amino acids could be indicative of its role in having the apoptotic activity.

Table 4: H-bonds and salt bridges of Arg55

H-bonding	Salt Bridges
Arg55 (N)---(O) Phe58	Arg55 (NH1)---(OD1) Asp57
Phe58(N)---(O)Arg55	Arg55 (NH2)---(OD1) Asp57
Arg55 (NE)---(OD1) Asp57	Arg55 (NH1)---(OD2) Asp57
Arg55 (NH2)---(OD2) Asp57	Arg55 (NH2)---(OD2) Asp57

It has been demonstrated that the interaction of enzyme with substrate to be highly dependent on the sequence and length of synthetic substrates. It is reported that GrB has an absolute requirement to cleave C-terminal to aspartic acid residues due to Arg226 in its S1 pocket (Waugh et al., 2000). A study of Jennifer et al (2000) found that substrate is due to the results of electrostatic surface formed by Arg192 and Arg226. Present study observed that in GzmH

instead of Arg192 and Arg226 the amino acid Ser217 and Lys231 may shows the substrate specificity. Among these Ser217 is near to the catalytic triad (Asp108 & Ser202) having a distance of 3.3A, while Lys231 is near to Asp108 (an active center amino acid). Lys231 also form H-bonds and salt bridges with OD1 & OD2 of Asp169. However this finding needs to be confirmation. The possible H-bonding and salt bridges are tabulated (Table 5).

Table 5: The possible H-bonding and salt bridges of Ser217 and Lys231

H-bonding	Salt Bridges
Ser217 (OG)---(OD2) Asp108	Lys231 (NZ)---(OD1) Asp169
Ser217 (O)---(OG) Ser202	Lys231 (Nz)---(OD2) Asp169
Ser217 (N)---(O) Val228	
Lys231 (N)---(O) Thr184	
Lys231 (O)---(N) His234	

Granzyme substrate interaction

Substrate of GzmB, Poly (ADP-ribose) polymerase (PARP) is a eukaryotic nuclear protein involved in differentiation, DNA repair, and chromatin structure formation (Jennifer et al., 1998). It contains a conserved protease recognition site, DEVD, which is known to be a target for several caspases (Bots and Medema 2006). Based on the interaction of PARP with granzyme B, two potential granzyme B cleavage sites were identified in poly(ADP-ribose) polymerase, VDP(D↓S)G at position 536 in PARP and LEI(D↓Y)G at position 644 (Kam et al., 2000). As GzmH shows the highest sequence homology with GzmB (Hanson et al., 1990) the substrate PARP may be used to identify the activity of granzyme H. It is reported by Edward et al (1999) and Gusakov et al (2000) that GzmH is shown to have chymotrypsin-like thioester activity with a preference for hydrophobic, aromatic amino acid residues (Phe or Tyr) at the P1 site. It is possible that like GzmB the enzyme GzmH can cleave the potential sites of PARP VDP(D↓S)G at position 536 in PARP and LEI(D↓Y)G at position 644 and shows chymotrypsin like thioester activity.

In granzymes there is a segment of Ser(214)-Cys(220), which normally provides a template for substrate binding, bulges out of the active site and is distorted. In GzmH the segment of Gly214-Asn220 is present to the outer surface of ganzyme near the C-terminal region. It is observed that there is a net work of H-bonds of the Gly214, Leu216 and Ser217 with other amino acids including the amino acid of catalytic site (Ser202).

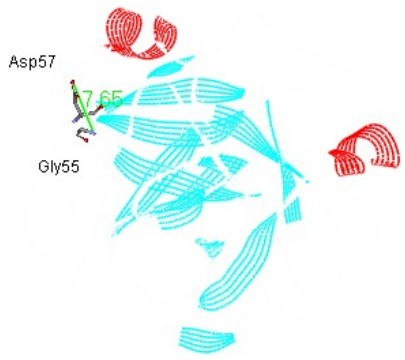


Fig Loss of H-bond between mutated Gly55 and Asp57

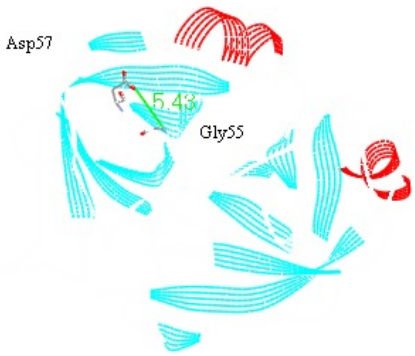


Fig Loss of H-bond between mutated Gly55 and Asp57

The other amino acid residue of segment like Tyr218, Gly219 and Asn220 shows surface accessibility (13.77 Å, 10.96 Å & 21.14A°). Among these amino acids the N atom of Gly214 forms H-bond with O atom of Leu206. The O atom of Ser217 forms H-bond with OG atom of Ser202. On the other hand Leu216 forms H-atom with Gly203 and Gly204. Hink-Schauer et al, 2002 suggest that this strand or segment will maintain its non-productive conformation in mature GzmK, mainly due to the unusual residues Gly(215), Glu(219), and Val(94). It is suggested that this segment for substrate upon approach transiently induce a functional active site confirmation. In GzmH these unusual residues are Phe235,

Ile239 and Asn104. Among these Phe235 forms 2 H-bonds. One with Val212 (N-O) and other with Ile239 (O-N). Asn104 forms H-bond (N-OD1) with Asn81. It is noticed that Asn104 shows a significant surface accessibility of 16.2495 having N-glycosylation site. The residue of Ileu239 form H-bond (O-N) with Met243. It is therefore found that besides these unusual amino acids, a segment of Gly214-Asn220 present near the catalytic triad of Gzm. The type of H-bonding and surface accessibility of amino acids of segment confirm that it may provides a template for substrate binding bulges out of active site and distorted. Possible H-bonding of this segment is figured & tabulated (Figure 3; Table 6).

Table 6: H-bonding in segment Gly214-Asn220

Gly214 (O)---(N) Leu186
Gly214 (N)---(O) Leu186
Ile215(O)---(N)Ile230
Ile215(N)---(O)Ile230
Leu216(O)---(N)Gly203
Leu216(N)---(O)Gly204
Ser217 (O)---(OG) Ser202
Ser217 (N)---(O) Val228
Tyr218 (N)---(O) Val228
Tyr218 (OH)---(OD1) Asn179
Ser217 (N)---(O) Val228

In GzmK, Ile215, Gly219 and Arg 84 normally provides a template for substrate binding and this segment for substrate upon approach transiently induce a functional active site confirmation (Hink-Schauer et al, 2002). It is observed that in GzmH, Arg84, Gly219 having an accessibility of 70.4973& 10.96A respectively present near the catalytic triad. Among these Gly219 more closely related to catalytic triad. No H-bond of both these amino acid observed. However, Ileu215 form two H-bonds with Ileu230. It may be possible that the highly accessible Arg84 and the presence of Gly219 near the catalytic triad may provide a template for substrate binding in GzmH. This shows that GzmH having some functional similarity with GzmK.

Presence of hydrophobic residues at the surface may have stability property by presuming a shield and also provide a site for enzyme substrate interaction. A hydrophobic patch of Trp, Leu, Phe, Ileu present at C-terminal, is catalytically significant and play a role in determining substrate interaction site (Strub et al., 2004). In GzmH, the hydrophobic patch from Trp238, Ile239, Lys240, Arg241 present at the surface of enzyme in helical form. The amino acid residues of this patch forms H-bonds with both polar and non- polar amino acid present in their primacies. Among these Lys240 and Arg241 shows a high surface accessibility (35.51 Å & 55.31 Å). The high surface accessibility and H-bonds with polar and non-polar amino acids demonstrate that this site of GzH may play a role in enzyme substrate interaction. Possible H- bonds of this helical patch is tabulated in (Table 7).

Table 7: H-bonding in helical patch Trp218-Arg221

Trp238 (O)---(N) Thr242
Trp238 (O)---(OG1) Thr242
Ile239(N)---(O) Phe235
Ile239(O)---(N) Met243
Lys240 (N)---(O) Leu236
Lys240 (O)---(N) Lys244

Besides the hydrophobic patch, a hydrophobic pocket is also formed in GzmB. The pocket is consisting of Ile-99, Tyr-215, and Tyr-175 and around the P4 position of the substrate (Waugh et al., 2000). In GzmH instead of Ileu99, Tyr215 and Tyr175, hydrophobic pocket may form from Ala99, Leu175 and Ilu 215. The surface accessibility of Leu175 is 29.2769 Å and of Ala99 is 15.1465 Å. It is also reported that substrate can make favorable hydrophobic interactions with Ile-35, the Cys-42Cys-58 disulfide bond, and the aliphatic portion of Lys-41 (Waugh et al., 2000). A substrate –hydro-

phobic interaction was also studied in GzmH. It is observed that in GzmH instead of Ileu35 there is Met35 and a disulfide bond of Cys49-65. It is noted that Met35 and Leu41 form H-bonds (N---O) with basic residue of Arg32 and Ser69 respectively. It is also observed that Met35 the Cys-49Cys-65 disulfide bond, and the aliphatic portion of Leu-41 form H-bonds and hydrophobic interaction with catalytic triad. It is therefore possible that these residues may show hydrophobic interactions with substrate when in binds with active center of GzmH.

Arginine 192 appears to be important in translating the extended substrate binding interactions into specific catalysis (Harris et al, 1998). Another study found that Arg 192 enhances the basic character of S1 and may interact with the substrate directly or through the ordered water molecule 407 (Waugh et al., 2000). A study postulated that arginine 192 plays a synergistic role with arginine 226 in determining the P1-aspartate and P3-glutamate substrate specificity (Harris et al, 1998). The present study observed Lys192 instead of Arg192 in GzmH. Lys192 shows a highest accessibility of 55.95. It forms H-bond (N-OD1) with Asp190. It is possible that like other granzymes due to the high accessibility and basic character, Lys192 may interact directly with substrate. In granzyme H instead of Arg226 the Pro is present at position 226. No H-bond of Pro226 is observed. However, according to the study of Waugh et al (2000) , it may be possible that this Pro residue in granzyme may orient the scissile bond for transition-state binding of substrate.

In family of granzymes (serine proteases), the side chains of the aromatic residue 215 fix the exposed β11 template segment to the underlying strand β12 of enzyme. Simultaneously, this residue provides the basis for subsites S2 and S4. However, it is found that Gly215 of GzmK cannot substitute for an aromatic residue and, rather, might push His57 out of its catalytic position(Ruggleset al., 2004) . On the other hand, GrB requires extended substrates for catalysis that span five recognition sites. S4 is defined by Ilue 215, Arg 174, Ala 99 and Leu 171, and favors -branched hydrophobic amino acids. S3 is defined by Arg 192 and Asn 218, and favors long polar side chains. At S2, the side chain is primarily exposed to solvent but is also bracketed by Ile 99 and His 57 (Waugh et al., 2000). Present study tried to find out the extended substrates for catalysis that span five recognition sites in GzmH. It is observed that the S4 is defined by Tyr 215, Tyr 174, Ile 99 and Gln 171, and favors -branched hydrophobic and hydrophilic amino acids. S3 is defined by Lys 192 and Trp218, and favors long polar side chains. At S2, the side chain is primarily exposed to solvent but is also bracketed by Ala99 and Asp57. In GzmH there is branched residue of Ile215, which form two H-bonds with Ileu230 (N-O & O-N). This branched residue may provide the basis for subsides S2 and S4 like trypsin related active serine proteases. However it needs a confirmation.

Mutation Prediction

An alteration in the sequence of purine and pyrimidine bases in a gene due to change, removal or insertion of one or more bases may result in an altered gene product (Murray et al., 2000). Several mutations affected the production of the protein, most probably reflecting their influence on folding efficiency. Mutations to hydrophilic residue may disrupt the bond and thus decrease the probability of the protein reaching its active conformation. If a hydrophobic residue is less exposed to the solvent in the denatured form than in the native form, it will oppose folding (Pakula et al., 1990). Some mutations destabilize the protein. The presence of hydrophobic residues at the surface may have stabilization properties by providing a shield from penetrating water molecules. On the other hand mutation to charged residue may disrupt a hydrophobic bond (Van den Burg at al., 1994).

In the present study we mutated some amino acids of granzyme H by using the software SPDV viewer 3.7.

(i) Lys222→Ala222, Pro225→Arg225 mutants:

Bird et al (2005) proposed that in the “single” mutant, where cs2 was changed to D239AATMAAY showed significantly

impaired attachment of heparin. They also observed that GrB cs mutants have impaired perforin-mediated cytotoxicity. This mutation affects accumulation of GrB in the target cell. We mutated residue Pro225→Arg225 and Lys222→Ala222 of granzymeH. It was observed that residues Lys222 and Pro225 are present near the cationic site cs2 of granzyme H. Mutation of Lys222→Ala222 & Pro225→Arg225 leads to the change in the distance of mutated residues with cs2 which may affect the stability of cs2 (Table 8).

Table 8: Comparison of amino acid residues forming hydrogen bonds in the wild type/mutated (Lys222→Ala222, Pro225→Arg225) GzmH models.

Wild Type	Dist (Å)	Mutant GzmH	Dist (Å)
198Phe(N)-202Ser(O)	5.13	198Phe(N)-202Ser(O)	4.20
198Phe (N)-222Arg (NH1)	3.98	198Phe(N)-222Ala(O)	3.98
225Pro (N)-194Thr(OH)	5.05	225Arg (NH2)-194Thr(OG1)	2.25
225Pro (O)-195Gly(N)	4.76	225Arg (NH2)-195Gly(N)	3.12

The mutation of Lys222 to Ala markedly decreased the surface accessibility from 47.89Å to 16.99Å. According to Strub et al (2004), mutagenesis showed that hydrophilic amino acid present at the surface has stabilizing effect while placing a hydrophobic residue in a solvent exposed position causes destabilization. The mutation of Pro225 to Arg however, showed a slight change in surface accessibility from 3.47 Å to 4.04Å. It is stated that addition of positive charge may result in the addition of charge repulsion which decreases the stability (Gusakov et al., 2000). Another study reported that mutation to hydrophilic residue may disrupt the bond and thus decrease the probability of protein reaching its active confirmation et al (2004. In view of these studies, it is stated that this mutation in turn may affect the uptake of GzmH into target cells, cytoplasmic distribution with reduced accumulation in target cell, and slightly impaired cytotoxicity of GzmH.

(ii) Arg55→Gly55 &Arg116→Glu116 mutants

It has been proposed that Arg55 and S-S bond of Cys49-Cys65 may show an apoptotic promoting caspase activity (Harris et al., 1998). Arg55 is present near the cationic cs1 site, Leu114-Glu115-Arg116--Lys117-Ala118. This site forms a number of H-bonding and salt bridge interactions with other amino acids (Table 3). In the present study, it is predicted that the mutation of Arg55→Gly55 causes loss of H-bonds between mutated Gly55 to Asp57 (Fig 1 →1a,1b). The mutation of Arg to Gly also resulted in decreased surface accessibility from 28.90Å to 4.71Å (Table 8). It is noted that the presence of Arg55 near such an important site (cationic site) and its role in forming bonds with other amino acid may confirm it role in showing the apoptotic promoting activity. It is therefore possible that mutation of Arg may affect the cytotoxic activity of GzmH.

Arg116 is present in the cationic site (cs1) of granzyme H which may play role in the uptake of GzmH by the cells. Bird et al (2005) reported that mutation of cs1 results in a form of GrB that shows significantly reduced binding to heparin, reduced uptake into target cells and cytoplasmic distribution with no apparent accumulation in IGD. Mutation of Arg116 to Glu resulted in loss its H-bonds and salt bridges with Glu115 (Fig 2→2a,2b). The mutation also decreased the surface accessibility from 35.11Å to 6.29 Å (Table 9).

Table 9: Comparison of amino acid accessibilities (A^0) in the mutated residues in the predicted model of granzyme H

Amino acids	Accessibility Wild type	Accessibility Mutant
Lys222/Ala	47.89	16.99
Pro225/Arg	3.47	4.04
Asn179/Met	9.52	32.13
Asp210/Gly	31.18	12.90
Arg116/Gly	35.11	6.29
Arg55/Gly	28.90	4.72

This shows that the mutation of both Arg55 and Arg116 affect the cytotoxic activity of GzmH (Table 10 and 11).

Table 10: Comparison of amino acid residues forming hydrogen bonds in the wild type/mutated (Arg55→Gly) GzmH models.

Wild Type	Dist (Å)	Mutant GzmH	Dist (Å)
55Arg (N)-58Phe (O)	3.26	55Gly (N)-58Phe (O)	3.23
55Arg (NH2)-57Asp (OD2)	3.06	55Gly (N)-57Asp (OD2)	7.65
55Arg (NH2)-57Asp (OD1)	3.40	55Arg (NH2)-57Asp (OD1)	5.43

Table 11: Comparison of amino acid residues forming hydrogen bonds in the wild type/mutated (Arg116→Gly) GzmH models.

Wild Type	Dist (Å)	Mutant GzmH	Dist (Å)
116Arg (NH1)-115Glu (OE1)	4.31	116Gly(N)-115Glu (OE1)	6.24
116Arg (NH2)-115Glu (OE1)	6.24	116Gly (NH1)-115Glu (OE1)	2.63
116Arg (NH1)-115Glu (OE2)	5.34	116Gly(N)-115Glu (OE1)	4.19

(iii) Asn179→Met179 mutant

In gzmH, Asn179 is an important N-glycosylation site which may play an important role in recognition of substrate. This mutation resulted in increase in the surface accessibility of mutated Met179 from 2.84 Å^2 to 3.68 Å^2. Mutated Met179 loses the H-bond with Tyr218. This mutation increased the surface accessibility from 9.52-32.13 A. According to a study Tyr218 form H-bond with Val228 and may act as a template for substrate binding (Hink-Schauer et al., 2002). The proposed mechanism may be that the Asn179 act as a recognition site for substrate bonded with Tyr218 which in turn act as template for substrate binding. This mutation therefore may fail to provide a space to substrate for binding with enzyme (Table 12).

Table 12: Comparison of amino acid residues forming hydrogen bonds in the wild type/mutated (Asn179→Met) GzmH models.

Wild Type	Dist (Å)	Mutant GzmH	Dist (Å)
179Asn (N)-179Asn (OD1)	2.84	179Met (N)-179Asn (OD1)	4.11
179Asn (O)-218Tyr (OH)	3.42	179Met (O)-218Tyr (OH)	5.36

(iv) Asp210→Gly210 mutant

This mutation may cause the loss of salt bridge between Asp210 and Arg32. Arg32 form H-bond with Met35 which is near Tyr34 (a residue that shows chymotrypsin like activity). Asp210 is present on the surface (accessibility = 31.1840 Å^2) of the GzmH molecule (Table 8) and is near the cs2 binding site of GzmH. This mutation may affect the H-bonding pattern of cs2 which may reduce binding to heparin, slightly reduced uptake into target cells, cytoplasmic distribution with reduced accumulation in cell and in turn may impaired cytotoxicity (Table 13).

Table 13: Comparison of amino acid residues forming hydrogen bonds in the wild type/mutated (Asp210→Gly) GzmH models.

Wild Type	Dist (Å)	Mutant GzmH	Dist (Å)
210Asp (OD1)-32Arg (NH2)	6.97	210Gly(O)-32Arg (NH2)	7.87

CONCLUSION

The predicted 3D model of human GzmH shows remarkable similarity with human GzmB in the overall 3D fold. However, significant differences in the X-ray structure and the protein model lie at the important functional sites. In the crystal structure of GzmB the catalytic triad is His57, Ser195 and Asp102 while in GzmH the catalytic triad is His64, Ser202 and Asp108. GzmH have cationic binding sites that may play a role in cytotoxic function of cell. Among cationic sites cs1 is Arg116, Lys117, Ala118, Lys119, Trp120, Thr221, Thr222, Ala223 that represent the heparin binding motif. The other cationic site is cs2 which consists of Lys172, Lys173, Thr174, Gln175, Thr176, Gly177, Pro178. This site binds glycosaminoglycan and enters into the cell cytoplasm. An ideal peptide mainly consisting of amino acids Leu114, Glu115, Arg116, Lys117, Ala118 is a part of cs1 site. The peptide may promote the conversion of procaspase to caspase which successively cause cell death. A segment of Gly214 to Asn220 is present near the catalytic triad of GzmH. This segment may provide a template for substrate binding and bulges out the active site. This segment maintains its conformation due to the presence of Phe235, Ileu239 and Asn104. A hydrophobic patch of Trp238, Ileu239, Lys240 and Arg241 is present in the helical form that provides a site for enzyme substrate interaction. Mutation of the amino acid residues, Arg55 and Arg116 may affect the cytotoxic activity of GzmH. It is concluded that identification of granzyme H mutations, or altered expression in human disease, would definitively place granzyme H on the list of key cytotoxic granule effectors molecules.

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