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Homology Modeling of Nematode *Caenorhabditis elegans* CED3 Protein–Inhibitor Complex

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CED3 protein, the product of a gene necessary for programmed cell death in the nematode *Caenorhabditis elegans,* is related to a highly specific cysteine protease family i.e., caspases. A tertiary-structural model has been constructed of a complex of the CED3 protein with tetrapeptide-aldehyde inhibitor, Ac-DEVD-CHO. The conformation of CED3 protein active site and the general binding features of inhibitor residues are similar to those observed in other caspases. The loop segment (Phe380-Pro387) binds with the P4 Asp in a different fashion compared to caspase-3. The comparative modeling of active sites from caspase-3 and CED3 protein indicated that although these enzymes require Asp at the position P4, variation could occur in the binding of this residue at the S4 subsite. This model allowed the definition of substrate specificity of CED3 protein from the structural standpoint and provided insight in designing of mutants for structure-function studies of this classical caspase homologue.

Key Words: **CED3 protein; *Caenorhabditis elegans;* caspase; apoptosis; programmed cell death; caspase-3; homology modeling; structure prediction.**

The caspase family of cysteine proteases plays a key role in the execution of apoptosis in mammalian cells (1–8). These proteolytic enzymes are characterized by the near absolute primary specificity for aspartic acid in the position P1 but notable differences of specificities in P2–P4 positions have been observed for individual members of the family (9–11). A caspase-related enzyme, the CED3 protein from nematode *Caenorhabditis elegans* (12, 13) is synthesized by a single transcript in a 56 kD proenzyme form (503 amino acid residues), which undergoes proteolytic activation to generate two subunits of 17 and 13 kD (designated by p17 and p13, respectively) (13). The CED3 protein has played a historical role in recognition of the involvement of the caspases in the programmed cell death because of its known central role in *Caenorhabditis elegans* apoptosis (14). This protein is closely related to caspase-3 according to its sequence similarity and substrate preferences (9, 15–17). It is demonstrated that the CED3 protein preferably inhibited by caspase-3-specific tetrapeptide inhibitor, Ac-DEVD-CHO (16). Moreover, the combinatorial studies showed that both the CED3 protein and caspase-3 preferentially cleave DE(T/V)D-X sequences (9). The overall sequence identity between CED3 protein and caspase-3 is 35%, whereas sequence identities between large subunits (p17) and small subunits (p12:caspase-3 and p13: CED3-protein) of both enzymes are 33 and 36%, respectively. The comparison of p17 of CED3 protein with the corresponding subunit of caspase-3 was made easier through a number of conserved residues (Fig. 1a). The pentapeptide 283QACRG287 and the tripeptide 236SHG238 segments, which possess the catalytic residues Cys285 and His237 of caspases are present in both sequences. The Arg179, one of the two arginines, which interact with the P1 Asp residue of substrate/

Amino acid residue numbers are those used in caspase-1 crystal structures (13, 14) (also known as ICE: Interleukin β-1 converting enzyme) as in the article describing the caspase-3 crystal structure (20); caspase-1 numbering has been mentioned.

Nomenclature for the substrate/inhibitor amino acid residues is Pn, . . . P2, P1, P1′, P2′, . . . Pn′, where P1–P1′ denotes the scissile bond. Inhibitor amino acids extending away from the scissile bond towards the amino terminus are denoted P1, P2, . . . Pn, whereas those extending towards the carboxyl terminus are denoted P1′, P2′, . . . Pn′. The corresponding binding sites in the enzymes are denoted S1, S2, . . . Sn, and S1′, S2′, . . . Sn′, respectively (37).

Abbreviations used: Ac-DEVD-CHO: Acetyl-Asp-Glu-Val-Asp-Aldehyde; PDB: Protein data bank; RMSD: Root mean square deviation.

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[2] This paper is dedicated in the memory of Professor Zafar H. Zaidi, the Vice Chancellor (President) of the University of Karachi, who passed away while in office on January 7, 2001. Professor Zaidi was an internationally renowned protein scientist. He pioneered protein chemistry in Pakistan and had 22 Ph.D.'s and more than 100 publications to his credit. He was a member of various scientific organizations of national and international repute. The Government of Pakistan had bestowed upon him several civil awards in recognition of his contributions to science and technology.

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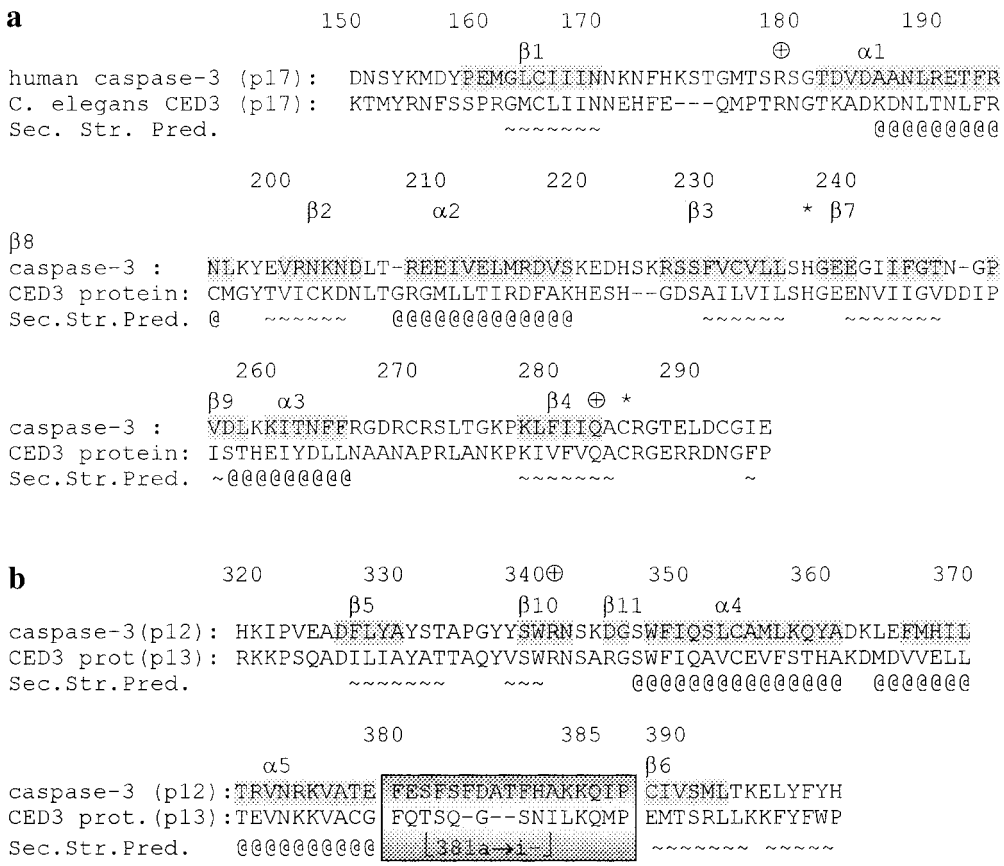

FIG. 1. The structure-based sequence alignment of Caspase-3 and CED3 Protein used for homology model building. (a) Large subunit "p17" of both proteins; (b) small subunit p12;Caspase-3 and p13;CED3 Protein. The α helices $\alpha1$–$\alpha5$ and β strands $\beta1$–$\beta11$ in caspase-3 structure are enclosed in shaded rectangles. The secondary structure predictions for CED3 protein sequence are shown by @ and ~(@ for α helices and ~ for β strands). The loop segment (380–387) in small subunits of both proteins which donates residues that make up part of the S4 subsite enclosed in a shaded box. Asterisks indicate the catalytic residues. The two arginines and a glutamine residue involved in recognition of P1 Asp side chain are indicated by ⊕.

inhibitor is also conserved. The N terminal half of p13 have conservative changes without any gap(s) compared to p12 of caspase-3 (Fig. 1b). This part contains the conserved Arg341 (among the two Arg residues present at S1 subsite) together with other amino acid residues which form S2-S4 subsites. The C terminal half of this subunit contains a highly variable region (Phe380-Pro387) corresponding to the loop structure located between the helix $\alpha5$ and strand $\beta6$. The CED3 protein contains a three-residue deletion at this region compared to caspase-3.

The crystal structures of caspase-1 (18, 19), caspase-3 (20, 21), and caspase-8 (22, 23) have been reported. The overall heterodimeric structures of these enzymes represent a new structural class of cysteine proteases and exhibit a topology that is novel with respect to known protein structures. The present communication describes the homology model of the CED3 protein complexed with the tetrapeptide inhibitor Ac-DEVD-CHO with the objective to analyze the possible fingerprints for molecular recognition at the enzyme-inhibitor interface.

METHODS

Sequence analyses. Sequences of the CED3 Protein from *Caenorhabditis elegans* and caspases from human were obtained from SWISSPROT database (24). Homology searches of SWISSPROT (24) and PDB (25) were carried out either by FASTA (26) and BLAST (27). Multiple sequence alignment of full-length CED3 protein and caspases was carried out using CLUSTAL W (28). As a consequence separate multiple alignments for the large and the small subunits of the CED3 protein and human caspases were constructed. The secondary structure prediction of CED3 protein sequence was carried out using the PHD method (29) (at http://www.embl-heidelberg.de/predictprotein/).

Model building, refinement, and evaluation. The homology model of CED3 protein in complexation with tetrapeptide inhibitor Ac-DEVD-CHO was built using human caspase-3 crystal structures as template. All steps of homology model building and refinement were carried out by the protein structure-modeling program MODELLER (30). The input to the program was a structure-based pairwise sequence alignment of caspase-3 and CED3 protein (Fig. 1) along with the two crystal structure coordinate sets of human caspase-3:inhibitor complexes (PDB ids = 1PAU and 1CP3) (20, 21). The program was implemented using standard parameter sets and a database of proteins with known 3D structures. In order to scrutinize the reliability of the alignment and the modeling of variable surface loops,

structural investigations on the graphics screen using 3D visualization programs, WebLab Viewer (33) and RasMol (34) were performed. If adjustments in the alignment were necessary, further manual realignments were carried out. The output provided (without any user intervention), tertiary model(s) of the CED3 protein–inhibitor complex containing all main chain and side chain heavy atoms.

The ENERGY command of the MODELLER (30), the programs PROCHECK (31), and PROSA (32) were employed for the assessment of reliability of the homology models. In addition, the variability of the models was compared by superposition of Cα traces and backbone atoms onto the caspase-1 (18, 19) (PDB id = 1ICE) and caspase-3 (20, 21) (PDB id = 1PAU and 1CP3) crystal structures, from which the RMSD value for positional differences between equivalent atoms could be calculated.

RESULTS

Homology Model of CED3 Protein

The tertiary structure of CED3 protein model elucidated is remarkably similar to crystal structures of caspase-1 (18, 19) caspase-3 (20, 21), and caspase-8 (22, 23) (Fig. 2a). The RMSD between 227 equivalent Cα atoms of caspase-3 and CED3 protein is 0.4 Å. The heterodimeric form of CED3 protein is formed by the p17 and p13 subunits that are folded into a compact cylindrical structure that appears as a single domain. The protein fold of this dimer consists of a central six-stranded β sheet with five parallel and one antiparallel strand. Four β strands are contributed by p17 (β1–4) and lie parallel to one another. Remaining two β strands (β5 and β6) are from p13 and are ordered such that the first lies adjacent and parallel to β4, while the second and the final strand of the sheet runs antiparallel to the rest. This central six-stranded β sheet is roughly sandwiched by five helices (designated as α1–α5), three on one side and two on the other side of the main β-sheet. There are two small two-stranded antiparallel β-sheets (β8–9 and β10–11) present at the top and front of the molecule, respectively. The antiparallel β-sheet, β10–11, situated at the top of the structural core is the part of the active site.

The notable structural differences between the caspase-3 structure and CED3 protein model are due to the insertions and deletions in different loop regions (Figs. 1 and 2b). The CED3 protein has insertions of an amino acid in two loop regions compared to caspase-3; (i) between the secondary structures β2 and α2, and (ii) β8 and β9. It has deletions of two and three amino acid residues in the following three loops compared to caspase-3; (iii) three residues between β1 and α1, (iv) three residues between α5 and β6, and (v) two residues between α2 and β3.

The three residues deletion between α5 and β6 structures occurs in a loop segment (Phe380-Pro387) which forms a reverse turn over the bound inhibitor and contributes several residues that make up part of the S4 subsite (Fig. 2b). The deletions between β1 and α1 and α2 and β3 structures occur in the loops located in

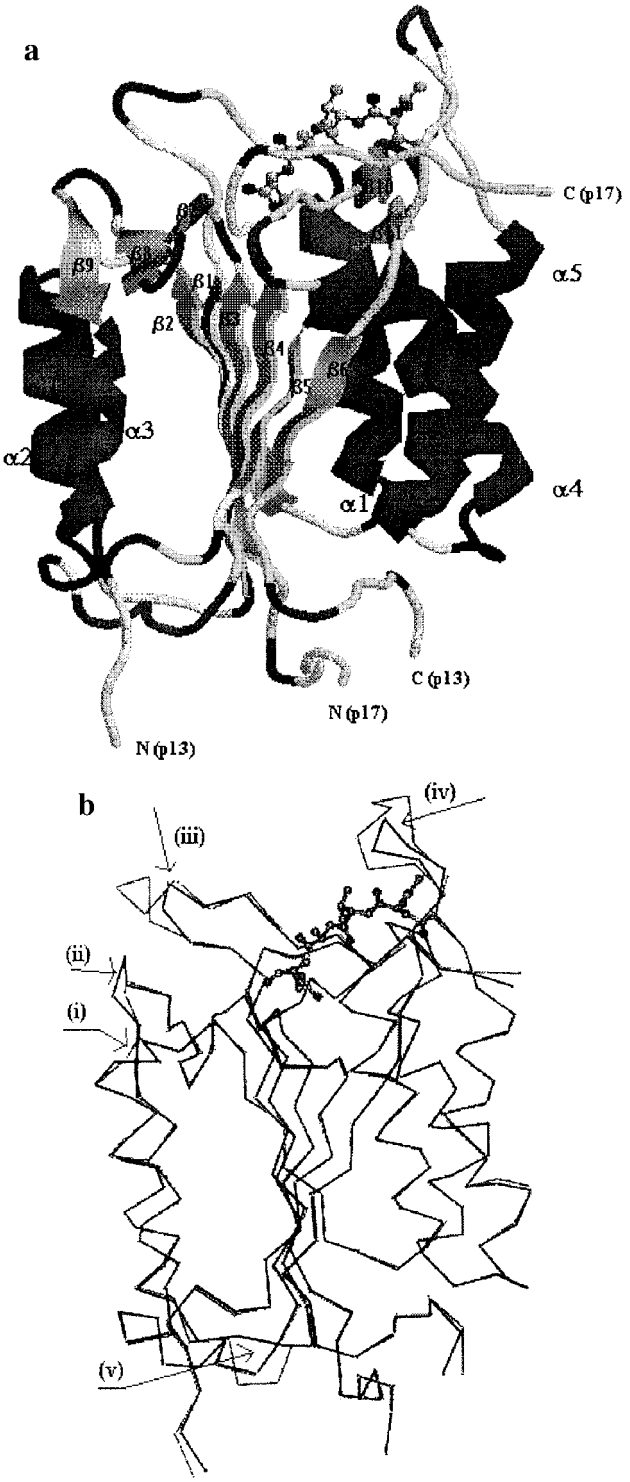

FIG. 2. (a) Schematic representation of CED3 protein:Ac-DEVD-CHO complex structure. The α helices are labeled α1–α5 and β strands are labeled β1–β11. The tetrapeptide inhibitor is located at the top center of the enzyme shown by a ball-&-stick drawing. (b) Structural superposition of Cα atoms of heterodimers of CED3 protein model (black) and the crystal structure of caspase-3 (grey). The structural differences due to the insertions and deletions in loop regions are indicated by arrows "i–v" (see Results).

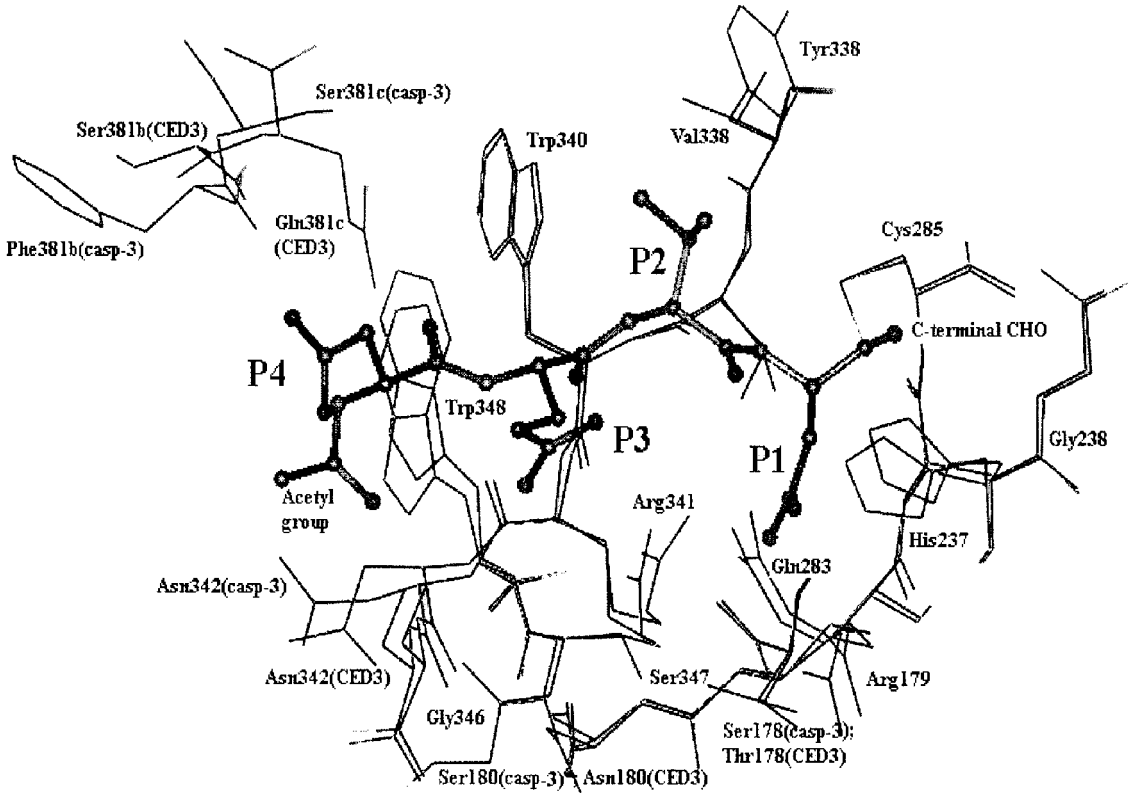

FIG. 3. The binding of peptide inhibitor Ac-DEVD-CHO on the active site surface of CED3 protein model. The CED3 protein residues those have at least one atom within 5 Å of any atom in the inhibitor are superposed on the equivalent residues of caspase-3 for highlight sequence and conformational differences. Amino acid residues of both proteins are depicted in line whereas residues of inhibitor (P1–P4) in ball-&-stick representation.

a region that is predicted to interact with the C-terminal part of the substrate. These structural variances in the CED3 protein at and near the active site is most likely responsible for differences in substrate recognition and binding on both sides of the scissile bond.

The Active Site of CED3 Protein Model

Figure 3 shows the binding of tetrapeptide inhibitor Ac-DEVD-CHO on the active site surface of CED3 protein model. The general binding features of Ac-DEVD-CHO at CED3 protein active site are in agreement with what is observed in caspase-1 (18, 19), caspase-3 (20, 21), and caspase-8 (22, 23) inhibitor complex structures.

The inhibitor adapts an antiparallel β strand like conformation by forming three main chain hydrogen bonds with β9 strand of p13 subunit. The main chain of residues Ser339 and Arg341 form three H-bonds with main chain of P1 Asp and P3 Glu, respectively. Both of the residues (e.g., Ser339 and Arg341) which participate in H-bonding to inhibitor are almost entirely conserved in all caspase sequences. This reflects the pivotal role of these hydrogen bonds in maintaining the binding of inhibitor on the CED3 protein active site surface (Fig. 3).

The modeling indicates a covalent bond linking the aldehyde of the inhibitor to the thiol of active site Cys285 can be formed for the CED3 protein:Ac-DEVD-CHO complex as observed in caspase-1 (18, 19) and caspase-3 (20, 21). The imidazole side chain of His237 is situated close to the residue Cys285 forming the catalytic diad of CED3 protein (Fig. 3). The C-terminal -CHO of the inhibitor is positioned such that it can form H-bonds with backbone nitrogens of Gly238 and Cys285 as well as with the ND1 of His237 side chain. The "oxyanion hole," which can stabilize the aldehyde oxyanion of the reaction intermediates formed during hydrolytic mechanism appears to be made by amide nitrogen atoms of Gly238 and Cys285 (20). The NE2 of imidazole ring of His237 is in H-bonding distance to the main chain nitrogen of Pro177 (caspase-3 has Thr at 177).

The S1–S3 Subsites

The S1–P1 binding site is situated at the "center" of the active site, which accommodates the P1 Asp side chain. The S1 subsite formed by the side chains of Arg179 and Gln283 from p17 subunit and by Arg341 and Ser347 from p13 subunit (Fig. 3). The chemical nature, 3D conformation, and interactions of these res-

idues with P1 carboxylate are remarkably similar to their counterparts in caspase crystal structures (18–23).

At the S2–P2 binding site, the Valine residue in position P2 of Ac-DEVD-CHO makes van der Waals contacts with Val338 and Trp340 amino acid residues of CED3 protein. Trp340 is conserved in both enzymes whereas Val338 in CED3 protein corresponds to a Tyr in caspase-3. The P2 Val side chain extends in contact with a hydrophobic surface of the S2 site and proximal to the side chain of Val338 (Fig. 3).

The S3–P3 binding site of CED3 protein is comprised residues Thr178, Asn180, and segment 341RNS343. Like the S1 subsite, residues from both subunits contribute to form S3 subsite (Fig. 3). The residues Thr178 and Asn180 of p17 subunit are in van der Waals contacts with of P3 Glu side chain. These amino acids are not conserved in CED3 protein compared to caspase-3 as at the both positions, serine residues are substituted in caspase-3. Nevertheless, these mutations might not affect the binding of P3 glutamyl residue because the side chains of these amino acids are directed away from the protein surface. The side chain of P3 residue can be seen as an extended solvent exposed conformation pointing away from the enzyme's surface. It is placed such that its carboxyl group could not form polar or ionic interaction(s) with the residues Arg341 and Ser343 present nearby.

The S4 Subsite

The S4–P4 binding site of caspase-1 and caspase-3 differs both in the nature of amino acids residues and three-dimensional structure (18–20). In caspase-1, the large and shallow hydrophobic S4 site preferentially accommodates a tyrosyl side chain, whereas the corresponding subsite in caspase-3 is a narrow pocket that closely envelopes the P4 aspartyl side chain. A homology model of caspase-4 constructed from the caspase-1 structure pointed out that residues constituting the S1–S3 subsites were identical, few differences are noted in the S4 site (35). The combinatorial studies revealed that the caspase-1, caspase-4, and caspase-5 could accommodate large aromatic/hydrophobic amino acid in the position P4 whereas the CED3 protein, caspase-3, and caspase-7 prefer Asp residue (9). Despite this reported identity in substrate specificity between caspase-3 and CED3 protein, there are variances in the components and conformation of S4 subsite of caspase-3 and CED3 protein (Fig. 4).

The S4 subsite of CED3 protein comprises two tetrapeptide segments (340WRNS343 and 381QTSQ381c) and the amino acid residue Trp348 (Fig. 4a). The segment 340WRNS343 and residue Trp348 are conserved where as the segment 381QTSQ381c corresponds to 381ESFS381c in caspase-3. The tetrapeptide 381QTSQ381c is the part of the loop region (Phe380-Pro387) which forms a reverse turn over the P4 residue of the bound inhibitor (Figs. 1b, 2a, and 3). The residues comprising S4 subsite surround the P4 aspartic acid from the three sides. As is seen in solvent accessible surface representation (Fig. 4b) that the P4 side chain in CED3 protein is situated in a sterically less constrained environment compared to caspase-3 due to the mutation of Phe381b(caspase-3) → Ser381b(CED3 protein) and conformational difference of Asn342 side chain. The observed polar interaction between Asn342 and P4 Asp in caspase-3 could not be form in the CED3 protein because the Asn342 side chain is directed away from the interior of the S4 subsite (Figs. 3 and 4a).

The main chain and side chain atoms of P4 residue is predicted to form two H-bonds with the residues Ser381b and Gln381c of the segment 381QTSQ381c. The P4 Asp carboxylate forms a hydrogen-bonding interaction with the backbone nitrogen of Ser381b (Fig. 4a). The comparable interaction in caspase-3 (20, 21) is between the P4 carboxylate and main chain of Phe381b. The amino acid residue Gln381c with its extended side chain covered the backbone of P4 residue and formed another hydrogen bond between its side chain nitrogen ND2 and the main chain oxygen of P4 residue. This type of interaction has not been observed in caspase-3 structures (20, 21).

DISCUSSION

It is well documented that the caspase-3 is more closely related mammalian enzymes with CED3 protein both in terms of sequence similarity and substrate specificity (9, 15–17). The homology model of CED3 protein showed a very similar protein fold compared to other members of caspase family with experimentally determined structures (18–23). Almost all the structural variations between CED3 protein and caspase structures are concentrated at and around the active site and result in the differences in substrate/inhibitor recognition and binding.

It has been reported that aside from the absolute requirement for Asp at P1 the position P4 is another specificity determinant for caspases and caspase-related enzymes. The comparative investigations of the caspase-3 structure and CED3 protein model revealed that compared to caspase-3 the P4 Asp residue of Ac-DEVD-CHO differentially associated with S4 subsite of CED3 protein. The variation in the P4–S4 interactions appears to be due to the surface loop section (Phe380-Pro387). Amino acid residues Ser381b and Gln381c located in this loop predicted to form two hydrogen bonds with the P4 Asp residue. These analyses depict the role of the loop (Phe380-Pro387) in the binding of P4 residue. This surface loop region is one of the structural differences between the caspase-1 and caspase-3 subfamilies of caspases (CED3 protein belongs to caspase-3 subfamily (1, 20, 36)). All members of

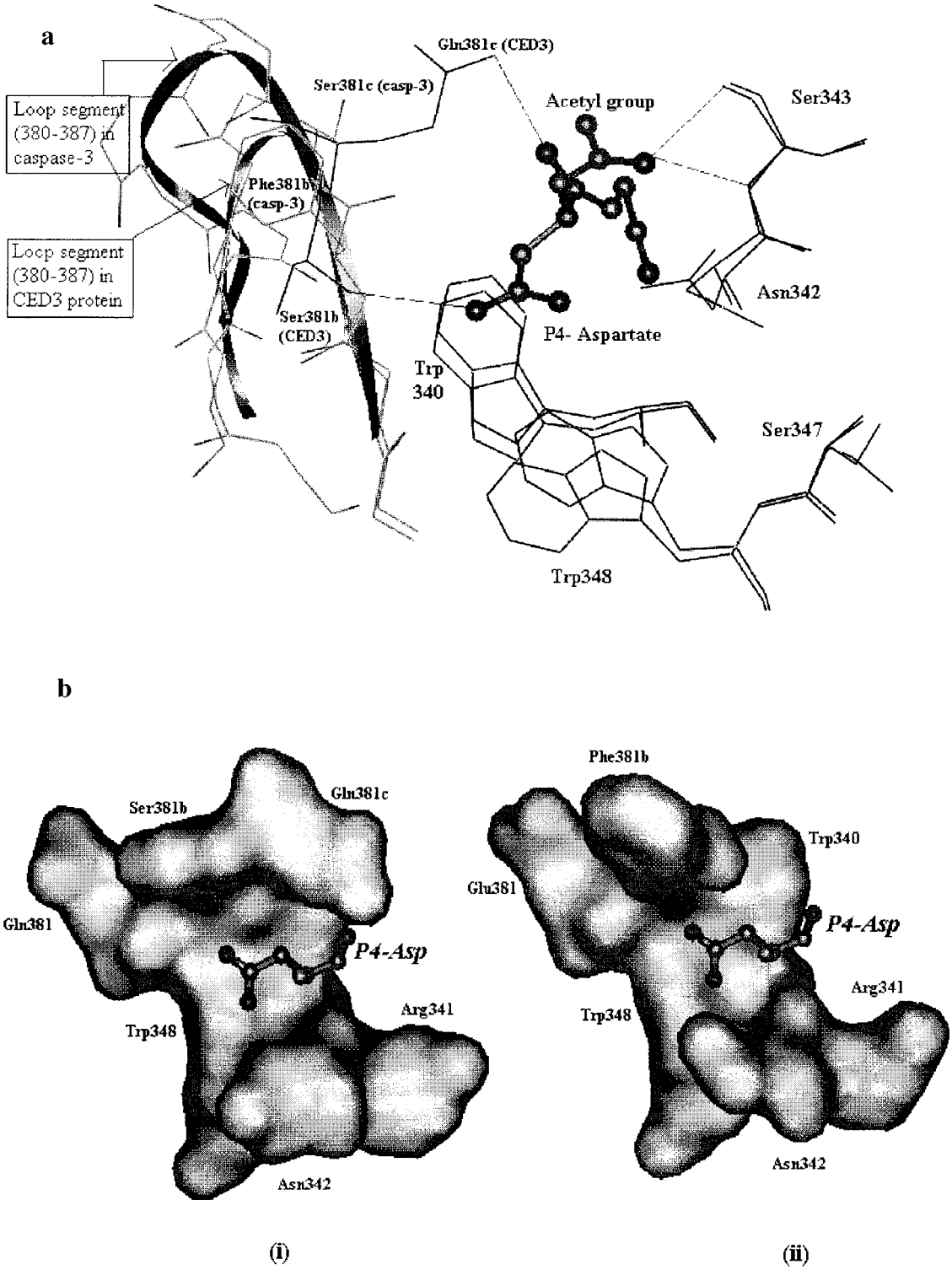

FIG. 4. (a) The S4 subsite of CED3 protein. The S4 subsite residues of CED3 protein superposed on the equivalent residues of caspase-3 depicted in line and P4 Asp of inhibitor in ball-&-stick representation. The reverse turn in the variable loop (380–387) is highlighted by ribbon in both structures. Dashed lines indicate hydrogen bonds. (b) Solvent accessible surface representation of S4 subsite residues in (i) CED3 protein and (ii) caspase-3.

caspase-1 subfamily are predicted not to contain this loop region while members of caspase-3 subfamily have an equivalent loop (20). Sequence analysis showed that the amino acid content and length of this loop region is different in all caspase-3 subfamily-related sequences (i.e., caspase-3, -6–10, and CED3 protein (1, 36)).

Therefore, it can be contemplated that each member of this subfamily could have different set of residues defining S4 subsite and the P4 residue of substrate/ inhibitor would have different modes of interactions with the corresponding subsite.

Modeling studies presented here indicate that amino acid residues of the loop (Phe380-Pro387) might interact with the P5 side chain of protein substrates. Therefore, along with the DEVD substrate recognition by CED3 protein, the loop region might take part in the event of preferentially recognition of protein substrates as well.

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