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A 3D-structural model of memapsin 2 protease generated from theoretical study[1]

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KEY WORDS Alzheimer disease; amyloid β-protein; templates; drug design

ABSTRACT

AIM: To build a 3D-structural model of memapsin 2 (M2) protease for theoretical study and drug design. **METHODS**: Structural alignment was performed based on multiple and pairwise sequence alignment of three templates. After the initial model was generated, energy minimization was completed by applying molecular mechanics method. Molecular dynamics (MD) technique was used to do further structural optimization. **RESULTS**: The 3D-structural model of memapsin 2 was constructed. The model is reasonable according to several validation criteria. The active-site motifs of M2 are structurally supported by a β-sheet rich domain and linked together with this domain through α helices. Tyr132 contained in β-hairpin is a general characteristic of aspartic protease. The Cα atom superimposing result is a direct verification that M2 is structurally unique but still belongs to the aspartic protease superfamily. **CONCLUSION**: The 3D-structure model from our study is informative to guide future molecular biology study about M2 and drug design based on database searching.

INTRODUCTION

Alzheimer disease is characterized by the progressive formation in the brain of insoluble amyloid plaques and vascular deposits consisting of the 4 kD amyloid β-peptide (Aβ)[1]. Aβ occurs in two predominant forms with different COOH-terminal, Aβ40 and Aβ42. Overproduction of Aβ42 has been suggested to be the cause of familial early-onset Alzheimer's disease. Formation of Aβ requires proteolytic cleavage of a large type-1 transmembrane protein, the β-amyloid precursor protein (APP)[2,3]. To initiate Aβ formation, β-secretase cleaves APP at the NH_2-terminus of Aβ to release APPsβ, an about 100 kD soluble NH_2-terminal fragment, and CTF99, a 12 kD COOH-terminal fragment which remains membrane bound[4]. In an alternate pathway, α-secretase cleaves within the Aβ sequence, thus precluding the formation of Aβ. Cleavage by α-secretase produces a large soluble NH_2-terminal fragment, APPsα, and a 10-kD membrane-bound COOH-terminal fragment, CTF83[2]. Both CTF99 and CTF83 can be further cleaved by one or more γ-secretases, leading to the release and secretion of Aβ and the nonpathogenic p3 peptide respectively (Fig 1).

The emergence of human gene sequences in the expressed sequence tag (EST) database represents an important new resource for identifying novel human enzymes. Initially identified from the human EST database, the cDNA of two new human membrane-associated aspartic proteases, memapsin 1 (M1) and memapsin 2 (M2), have been cloned and sequenced[5-7]. These two proteases are unique among aspartic proteases superfamily[8], and the M2 is different from M1 in that it mainly exists in brain and fits all of the criteria of β-secretase. Furthermore, expression of APP and M2 in HeLa cells shows that M2 catalyzes the rate-limiting step of the progression of Alzheimer's disease, and thus, may be a strong therapeutic target[9].

In this paper, we have performed a 3D-structural modeling of memapsin 2 based on the general characteris-

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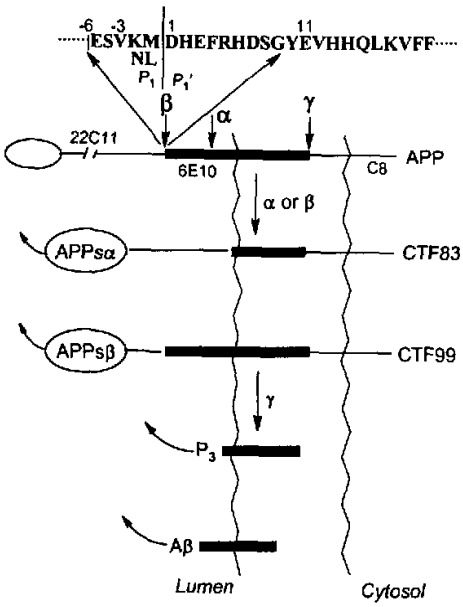

Fig 1. Illustration of proteolytic processing sites and cleavage products of APP. Wavy lines represent the cell membrane. 22C11, 6E10, and C8 are epitopes. Processing at the α-secretase site cleaves the mid-region of the Aβ sequence and liberates the APPsα ectodomain containing 6E10 epitope, whereas the consequent 83-amino acid C-terminal fragment (CTF83) retains the APP membrane domain (TM). Processing of the β-secretase site releases the APPsβ ectodomain and creates a 99-amino acid C-terminal fragment (CTF99) containing the 6E10 epitope. The amino acid sequence around the β-secretase site is numbered relative to M2. The position of the Swedish APP mutation, a substitution of P_1: Lys → Asn and P_1': Met → Leu, is indicated.

tics of 3D-structures of aspartic proteases. Sequence alignment (Fig 2) of catalytic domain of M2 with pepsin shows that it has the same two active-site aspartic acids in D(T/S)G motifs and a β-hairpin structure containing conserved Tyr residue, as that of aspartic protease superfamily. In the absence of high-resolution 3D-structure of M2, our model is a useful tool to study the cleavage mechanism at β-secretase site and to design new inhibitors targeting this new protease.

COMPUTATIONAL METHODS

The modeling process was done mainly in two stages, which included the generation of the initial model and the refinement of the structural model.

Generation of an initial model The first step in this procedure was to produce an initial sequence alignment of the target M2 with three templates. Their PBD entries were 1AM5, 3CMS, and 1QRP. These structural templates are found in the PDB database through Swiss-Model server (http://expasy.hcuge.ch/swiss-mod/SWISS-MODEL.html). 1AM5 is the crystal structure of a pepsin from Atlantic COD (*Gadus morhua*) at 2.16 Å resolution[10], 3CMS is an X-ray determined structure of chymosin B at 2.0 Å resolution[11], and 1QRP is a 3D-structure of human pepsin 3A complexed with a phosphonate inhibitor at 1.96 Å resolution[12]. Three components were used in this first step: one was a multiple sequence alignment based on the structural similarity among the templates themselves; another was a pairwise sequence alignment between 1QRP and M2 obtained from ALIGN program encoded in the FASTA program package; the final was the result from secondary structure prediction of M2 protease using SeqFold module in the InsightII software[13]. The final structural alignment among these three templates and the target is shown in Fig 2. Structural alignment conserved regions (SCR) are indicated just above the sequence in α helices and β sheets.

In the second step, the backbone coordinates of the residues in M2 were generated with InsightII/Homology module[13]. Regions in the M2 that aligned with the structurally conserved regions (SCR) among the templates were generated by copying the backbone coordinates from one of the templates, and the coordinates of 1QRP were used in this study. For the non-SCR regions, if the sequence length was the same as that of 1QRP, the backbone coordinates from 1QRP are used for M2. Otherwise, for the three longest loops indicated in Fig 2, a loop search of the structures in the PDB databank was performed. Usually, a maximum of 10 possible loop structures from PDB were chosen by InsightII/Homology and one of them, which had the lowest RMS squares and considerable geometrical compatibility with the SCR region, was selected visually as the template for the non-SCR in the initial model.

Finally, the two ends of the model were generated automatically through end-repair with InsightII/Homology[13]. During the process of generating backbone coordinates, initial side chain conformation for each residue was also generated automatically. Residues in M2 that were identical to those in 1QRP in the alignment were given identical side-chain conformations as in 1QRP. For non-identical residues, the conformations of these

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             1                                      MAQALPWLLLWM   12
                      *                                        #
            13  GAGVLPAHGTQHGIRLPLRSGLGGAPLGLRLPRETDEEPEEPGRRGSFVM  63

                ---β---  ---β---    ---------β--------    --α--
AM5          3  TEQMKNEADTEYYGVISIGTPPESFKVIFDTGSSNLWVSSSHCSAQACS  51
CMS          3  SVPLTNYLDSQYFGKIYLGTPPQEFTVLFDTGSSDFWVPSIYCKSNACK  51
QRP          3  EQPLENYLDMEYFGTIGIGTPAQDFTVVFDTGSSNLWVPSVYCSSLACT  51
M2          64  VDNLRGKSGQGYYVEMTVGSPPQTLNILVDTGSSNFAVGAA----PHPF 108

                -    --α--  ------β-----   -------β------
AM5         52  NHNKFKPRQSSTYVETGKTVDLTYGTGGMRGILGQDTVSVGGGSD--PN  98
CMS         52  NHQRFDPRKSSTFQNLGKPLSIHYGTGSMQGILGYDTVTVSNIVD--IQ  98
QRP         52  NHNRFNPEDSSTYQSTSETVSITYGTGSMTGILGYDTVQVGGISD--TN  98
M2         109  LHRYYQRQLSSTYRDLRKGVYVPYTQGKWEGELGTDLVSIPHGPN-VTV 156
                                                           ▲

                ----β----     --α--      ----α----  ----α----
AM5         99  QELGESQTE-PGPFQAAAPFDGILGLAYPSIAAAGAV--PVFDNMGSQSL 145
CMS         99  QTVGLSTQE-PGDFFTYAEFDGILGMAYPSLASEYSI--PVFDNMMNRHL 145
QRP         99  QIFGLSETE-PGSFLYYAPFDGILGLAYPSISSSGAT--PVFDNIWNQGL 145
M2         157  RANIAAITESDKFFINGSNWEGILGLAYAEIARPDDSLEPFFDSLVKQTH 206
                               ▲

                    --β---                      --β--  --α-|---β----
AM5        146  VEKDLFSFYL---------SGG-GANGSEVMLGGVDNSHYTGSIHWIPVT 185
CMS        146  VAQDLFSVYM---------DRN-GQESMLTLGAIDPSYYTGSLHWVPVTV 185
QRP        146  VSQDLFSVYL---------SAD-DQSGSVVIFGGIDSSYYTGSLNWVPVT 185
M2         207  V-PNLFSLQLCGAGFPLNQSEVLASVGGSMIIGGIDHSLYTGSLWYTPIR 255
                                 ▲

                     --β- --β-  -β-            ------β-----    ---
AM5        186  AEKYWQVALDGITVNGQTA--AC|EGC--QAIVDTGTSKIVAPVSALANI 231
CMS        186  QQYWQFTVDSVTISGVVVA--CE-GGC--QAILDTGTSKLVGPSSDILNI 231
QRP        186  VEGYWQITVDSITMNGEAI--ACAEGC--QAIVDTGTSLLTGPTSPIANI 231
M2         256  REWYYEVIIVRVEINGQDLKMDCKEYNYDKSIVDSGTTNLRLPKKVFEAA 305

                α---  -β-        -β-   ---α----  --β--              -
AM5        232  MKDIGASEN----|QGEMMG---NCASVQSLPDITFTI-----------N 263
CMS        232  QQAIGATQN----QYGEFDI---DCDNLSYMPTVVFEI-----------N 263
QRP        232  QSDIGASEN----SDGDMVV---SCSAISSLPDIVFTI-----------N 263
M2         306  VKSIKAASSTEKFPDGFWLGEQLVCWQAGTTPWNIFPVISLYLMGEVTNQ 355
                                                                ▲

                --β---|-α-|------β--------     --β-    ---α---|---
AM5        264  GVKQPLPPSAYIEGDQAFCTSGLGSSGVPS-NTSELWIFGDVFLRNYYTI 312
CMS        264  GKMYPLTPSAYTSQDQGFCTSGFQS-------E|QKWILGDVFIREYYSV 312
QRP        264  GVQYPVPPSAYILQSEGSCISGFQGMNLPT-ESGELWILGDVFIRQYFTV 312
M2         356  SFRITILPQQYLRPVEDVATSQDDCYKFAISQSSTGTVMGAVIMEGFYVV 405

                -----β--------
AM5        313  YDRTNNKVGFAPAA                                     326
CMS        313  FDRANNLVGLAKAI                                     326
QRP        313  FDRANNQVGLAPVA|VV|AA                               330
M2         406  FDRARKRIGFAVSACHVHDEFRTAAVEGPFVTLDMEDCGYNIPQTDESTL 455

                -----------TM----------
M2         456  MTIAYVMAAICALFMLPLCLMVCQWRCLRCLRQQHDDFADDISLLK     501
```

Fig 2. **Structural alignment of M2 protease with three other proteins 1AM5, 3CMS, and 1QRP, which belong to the aspartic protease superfamily. * : signal peptide cleavage site and the beginning of pro-memapsin region; # : the end of pro-memapsin and the beginning of mature memapsin region; α, β: the secondary structural types; ▲: the four predicted *N*-linked glycosylation sites in the M2 sequence; TM: the transmembrane domain of M2 predicted by hydrophobicity analysis. The two active-site aspartic acid in D(T/S)G motifs and the Tyr residue in the conserved β-hairpin motif are labeled black.**

side-chain atoms that were in common were copied from 1QRP to M2. For the remaining side chains, library values of rotamers were used and placed along the same direction as the aligned residue in 1QRP. An initial 3D model was thus completed.

Refinement of the initial model The raw structure of M2 was relaxed in all the SCR by using the Refine routine in InsightII/Homology[13]. Further structural modifications to the loops and side chain conformations of residues in the SCR were performed to avoid trapping of the loop in a high energy local minimum during energy minimization. In the minimizing process, first of all, the constraints of dihedral angles and hydrogen bond distances of the SCR's backbone were applied in the combining steepest descent and conjugated gradient minimization methods. After 5000 steps, these constraints were released gradually and removed during the full-protein optimization using the parameters as a nonbonding cutoff of 8 Å and a distance dependent radial dielectric $\varepsilon = 5r$, Amber force field and Amber-all-atom charges, the energy gradient RMS < 0.05 kcal (mol · Å)$^{-1}$. The whole model was minimized to convergence.

In the next step, the model was subjected to 40 ps molecular dynamics (MD) simulations using a long nonbonding cutoff distance of 15 Å. The model was kept at 300 K during the entire simulation. At the beginning of the MD, the same geometrical constraints as described above were used in order to keep the SCR regions of M2 structurally stable. The constraints were gradually reduced for a total of additional 5 ps and removed completely during the last 35 ps simulation process. The total energy of the model and the RMS (root-mean-square) deviation of the Cα atoms in SCR regions between two consecutive snapshots were monitored to evaluate the quality of MD operation and the structural stability of M2 protease. The averaged structure from the last 30 ps of the MD was then calculated and further optimized to generate the final refined structural model of M2 protease.

RESULTS AND DISCUSSION

Fig 2 represents the structural alignment of M2 with the four templates. This alignment was chosen not only according to the aligning score but also based on the conserved residues in the aspartic protease superfamily. The refined model of M2 protease was evaluated for its overall quality. First, the structural checking was performed using the ProtStat routine in the InsightII/Homology. Some structural flaws, such as appearance of hindrance or holes produced by incorrectly packed side chains, distorsions in incorrectly aligned regions, were found and corrected till that all the bond lengths and dihedrals were in the normal range of geometrical criteria. After that, the Profiles-3D routine in Homology module was applied to verify the residue environment according to the PDB classification in the smooth mode. The overall self-compatibility score of M2 protease was 123.45, and the score of every residue was above zero (Fig 3). Inspecting the results of secondary structure classification, all the per-residue secondary types agreed with the structural alignment (Fig 2) and the parameters setting by Kabsch-Sander algorithm[14]. That is to say, our structural model is in accordance with whole conformational arrangement of aspartic protease superfamily.

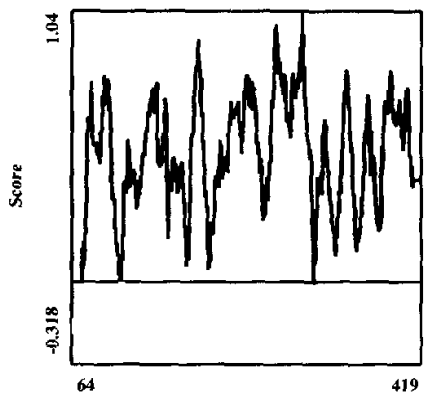

Fig 3. The 3D-profile map of M2 structural model.

Fig 4 represents the root mean square deviations (RMSD) of conformations of M2 during the molecular dynamics simulation. Although the solvent effect was not considered, a dielectric constant of 10 was adopted in the whole MD operation in order to simulate the *in vivo* environment. From Fig 4, we could see that the model was conformationally variable at the beginning of MD trajectory, but it became structurally stable about 10 ps later. These results provide an additional testification of the robustness of our model.

Fig 5 shows the cartoon map of the 3D-structural model of M2 protease, and Fig 6 is the representation of three highly conserved domains in the M2 model. Residue Tyr132 was situated in the conserved β-hairpin motif which was a general structure characteristic of aspartic protease. The distance between the phenyl ring center in the side chain of Tyr132 and Cα atom of Asp93

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was almost the same as that of the templates (Fig 6B). Although in agreement with structural criteria regarding other aspartic protease, the only β hairpin contained in the second conserved domain (Fig 6B) was a great difference existing in the subfamilies of aspartic protease. The two active-site motifs were structurally supported by the high conserved β-rich domain (Fig 6A). This means the region containing the active-site was highly conserving and actually the active catalytic site, which carries the cleavage function of β-secretase in the biological formation of Aβ during the process of Alzheimer's disease.

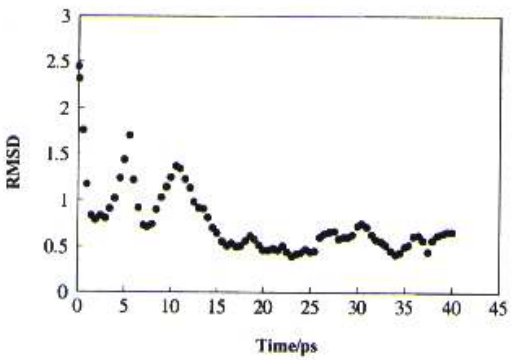

Fig 4. The root mean square deviations (RMSD) *vs* time map of different conformations of M2 in the molecular dynamics trajectory.

Fig 5. Cartoon representation of M2 protease 3D-structural model.

To check the familial onsets of M2 protease in biological evolution, atomic fitting about the active-site motifs was performed. The Cα superimposing map was generated and shown in Fig 7. Although the β hairpin containing region was somewhat conformationally changeable, the fitting results in Fig 7 coincide well with our expectation.

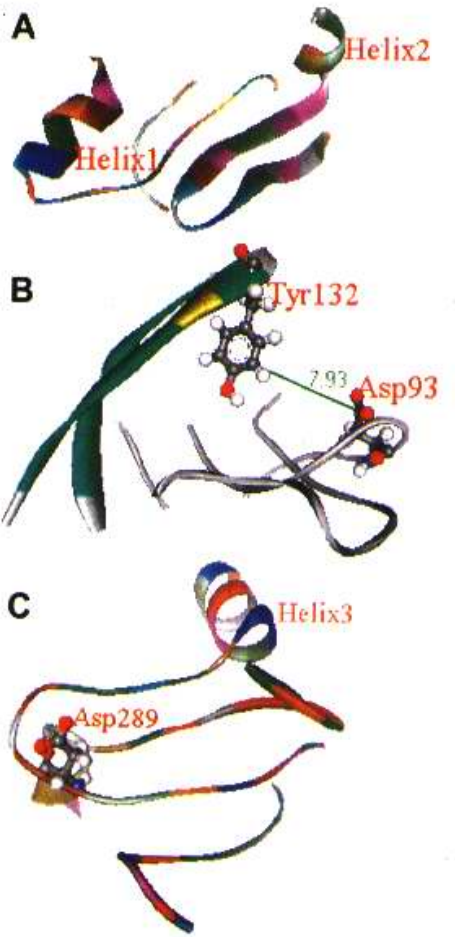

Fig 6. Cartoon representation of the three conserved domains in the structural model of M2 protease, which could also be found in the aspartic protease superfamily. A: the β-sheet rich domain just below the active aspartic acid motifs; B: the β-rich domain containing the first active-site motif, the distance between the Cα of Asp93 and the center of phenyl ring in the side chain of conserved Tyr132 is labeled; C: the third β-rich domain containing the second active-site motif. Helix 1 links the domain A and B, helix 2 links the domain A and C, helix 3 is another structural conserving motif between the domain C and the loop-rich region above the domain C.

Furthermore, the conformational measurement about the active-site motifs listed in Tab 1 (the templates and two additional aspartic protease from PDB bank) gives an ideal impression about the structural conservation of M2 protease.

Tab 1. The dihedral angles of the conserved active-site motifs of some aspartic proteases including the M2 protein.

Protein	Reisdue	phi	psi	Chi1	Chi2	Chi3	Chi4	zeta
	Asp32	-106.65	109.42	-177.38	26.37			33.14
	Thr33	-78.13	-12.39	71.30				29.80
	Gly34	-97.64	6.92					
1AM5	Tyr75	-105.28	33.41	-60.64	67.62	-179.41	-0.28	40.58
	Asp215	-113.72	112.81	-151.19	-32.61			43.76
	Thr216	-74.87	-21.58	52.17				34.41
	Gly217	-70.93	-20.87					
	Asp32	-127.01	98.26	178.19	18.90			35.92
	Thr33	-69.87	3.15	60.62				26.95
	Gly34	-109.88	18.30					
1EED	Tyr75	-117.95	178.43	-75.78	-77.95	179.74	2.23	41.31
	Asp215	-126.39	90.07	-171.35	-2.65			34.28
	Thr216	-53.27	-24.66	61.11				33.91
	Gly217	-83.97	5.84					
	Asp32	-114.88	110.06	-178.00	9.75			40.47
	Thr33	-76.98	-1.70	60.37				33.32
	Gly34	-99.23	6.11					
1HTR	Tyr75	-130.72	-173.98	48.56	76.72	-179.76	-0.04	28.18
	Asp217	-128.01	106.32	-171.33	-18.28			37.13
	Thr218	-74.55	-0.79	52.80				38.21
	Gly219	-96.33	8.00					
	Asp32	-110.81	111.17	-179.56	17.83			34.96
	Thr33	-82.50	3.25	61.90				31.11
	Gly34	-97.26	-2.31					
1QRP	Tyr75	-106.39	169.08	-53.26	-91.25	-177.84	0.33	33.20
	Asp215	-125.78	103.64	-172.76	173.01			33.39
	Thr216	-67.49	-17.36	56.70				33.58
	Gly217	-91.94	-7.39					
	Asp32	-112.91	93.78	-168.76	9.39			26.62
	Thr33	-74.14	-8.27	56.79				29.66
	Gly34	-95.40	7.49					
3CMS	Tyr75	-135.35	128.63	177.03	-79.45	-179.88	0.17	35.34
	Asp215	-121.36	110.60	-171.01	-4.22			36.97
	Thr216	-78.24	-2.43	63.28				35.68
	Gly217	-107.33	24.18					
	Asp93	-129.52	103.31	-179.39	-64.30			35.60
	Thr94	-80.67	9.33	58.69				32.58
	Gly95	-94.62	-24.59					
M2	Tyr132	-133.01	169.47	-58.76	-92.16	-179.88	-1.16	35.60
	Asp289	-114.49	95.00	172.64	-163.52			33.88
	Ser290	-51.83	-33.66	61.49	33.20			
	Gly291	-69.01	-35.06					

In conclusion, this work is the first to provide a validated 3D-structural model of M2 protease. The main goal of this work, using theoretical modeling methods to build a useful model structure for the structural study of cleavage mechanism of β-secretase acting on APP, has been fulfilled. Our model could allow structure-based selection of residues for future mutation studies to further probe the relationship between structural and biological function of M2 protease, and thus, offer a satisfactory drug design model based on small molecular database searching or *de novo* inhibitor designing.

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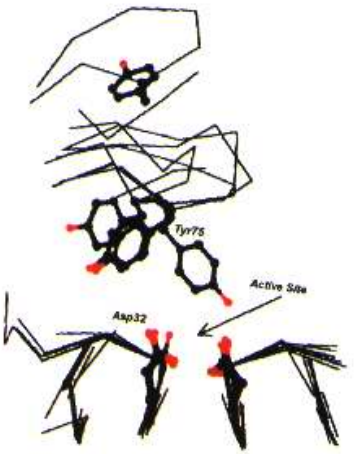

Fig 7. The Cα superimposing map about the conserved domains containing the active-site motifs and the Tyr residue in the β-hairpin motif of 5 aspartic proteases, extracted from the PDB bank with high sequence homology.

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用理论方法建立 memapsin 2 蛋白酶的三维结构模型[1]

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关键词 阿尔茨海默病；淀粉样 β-蛋白；模板；药物设计

目的：通过理论方法建立 M2 蛋白酶的三维结构模型． **方法**：基于模板进行多重序列联配和结构联配建立 M2 蛋白酶的初始结构模型，对初始模型进行分子力学和分子动力学优化，用多种评价方法对所得结构进行合理评价． **结果**：得到了 M2 蛋白酶的三维结构模型，合理性评价结果表明该结构模型正确，M2 蛋白酶的催化活性位点与天冬氨酸蛋白酶相似，区别在于与活性位点相邻的结构域的构象不同．结构保守区 α 碳原子叠合结果说明 M2 蛋白酶与其他天冬氨酸蛋白酶具有相同的生物进化来源． **结论**：M2 蛋白酶的结构模型可以为进一步的分子生物学实验提供有益的参考，也可以此模型为基础进行数据库筛选和药物分子设计．

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