THE CRYSTAL STRUCTURE AT 5.5Å RESOLUTION OF AN ACID-PROTEASE FROM RHIZOPUS CHINENSIS

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SUMMARY: An electron density map for the pepsin-like enzyme from Rhizopus chinensis has been calculated at 5.5Å resolution. The molecular boundary has been defined and certain secondary structural details have been inferred. The molecule is bilobal and has a large cleft in which several inhibitors have been observed to bind.

The acid protease from Rhizopus chinensis is a proteolytic enzyme, molecular weight 35,000, first isolated by Fukumoto et al. (1). The enzyme is active in the pH range 2-5, and has catalytic properties similar to pepsin, rennin and cathepsin D (2). It has a single polypeptide chain of about 330 amino acids, but only limited sequence information is available (3,4). In this report, we present a low-resolution (5.5Å) structure of the enzyme as determined by x-ray diffraction methods. Preliminary low resolution x-ray analysis has also been carried out on pepsin (5) and on two fungal acid-proteases from penicillium janthinellum (6) and Endothia parasitica (7).

Conditions for obtaining the crystals of the Rhizopus enzyme have been described earlier (8). The crystals are orthorhombic, with a unit cell of a = 60.3Å, b = 60.7Å, c = 107.1Å, spacegroup $P2_12_12_1$ and one molecule per asymmetric unit. The solvent content is about 56% (8). Heavy-atom derivatives were prepared by soaking the crystals in appropriate reagents

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and the data pertinent to these are given in Table 1. All intensity data were collected on a Picker FACS-I computer-controlled diffractometer. The major site of attachment in each derivative was located in difference-Patterson syntheses and were checked by cross difference Fourier calculations. Additional sites were picked up subsequently from difference-syntheses. The occupancies and positional parameters of the heavy atoms were refined by alternate cycles of phasing and least-squares refinement (10). The results of the refinement are in Table 1. The mean figure of merit for 1317 reflections is 0.92.

A three-dimensional electron density map at 5.5Å resolution was computed with the "centroid" phases (11) from the above derivatives. The molecular boundary could be clearly defined and revealed the enzyme to have the appearance of an elongated and somewhat flattened ellipsoid with approximate dimensions of 31 x 40 x 60 Å, Fig. 1a and 1b. These dimensions resemble those reported for other acid proteases (6,7). The molecules pack length-wise, in a "head-to-tail" fashion roughly along the c axis. It is only at the ends where two molecules are in contact that there is any question at all about the position of the molecular boundary.

In the middle of the ellipsoid, running roughly parallel to the 40Å axis of the ellipsoid is a deep groove (Figure 1a). It is in this groove that difference electron density maps have revealed the presence of the binding sites for two inhibitors of the enzyme, 1,2-epoxy-3(p-nitrophenoxy) propane (12) which has been shown to react with an active site aspartyl residue (Asp 32) in pepsin (13); and pepstatin (14) which is a strong competitive inhibitor of the enzyme (15). The epoxide was a gift from Dr. Jordan Tang. The pepstatin was received from Dr. Tang and also from Dr. H. Umezawa. It is also interesting to note that many of the heavy atom derivatives have one site located in this cleft (actually a matter of inconvenience from the point of view of the 'phase problem').

The molecule is divided into two lobes by the cleft and one of these

Table 1: Sites and occupancies of the various heavy-atom derivatives used in phasing calculations at 5.5 Å resolution

Heavy Atom	Site No.	x	y	z	O*	$\frac{RMS(\varepsilon)}{RMS(f_H)}$	R_c#	Heavy Atom	Site No.	x	y	z	O*	$\frac{RMS(\varepsilon)}{RMS(f_H)}$	R_c#
$K_3UO_2F_5$ (UF)	1	.090	.535	.045	88.4			Iodine	1	.213	.431	.471	37.8		
	2	.108	.562	.013	34.9	.57	.46		2	.430	.327	.489	43.9		
	3	.231	.516	.061	16.5				3	.122	.937	.457	33.3		
	4	.049	.707	.026	15.3				4	.501	.337	.457	44.2		
									5	-.014	.682	.396	35.6	.42	.43
$Pb(CH_3COO)_2$ (Pb)	1	.213	.515	.052	82.0				6	-.019	.361	.305	37.6		
	2	.124	.556	.019	35.0	.36	.42		7	.054	.907	.052	23.8		
	3	.368	.402	.486	18.1				8	.012	.874	.020	19.7		
	4	.314	.406	.214	11.7				9	.079	.910	.161	17.2		
									10	.279	.761	.152	13.2		
Pb+UF	1	.215	.511	.051	56.9										
	2	.312	.396	.178	43.0			Uranyl	1	.311	.394	.176	84.8		
	3	.045	.748	.035	30.8	.44	.43	Acetate	2	.214	.514	.050	71.3		
	4	.096	.533	.049	8.8				3	.062	.746	.042	32.3		
	5	.136	.575	.001	15.4				4	.150	.576	.003	21.0	.43	.48
Merbromin	1	.023	.561	.432	42.1				5	.130	.979	.408	19.6		
	2	.011	.138	.077	20.7	.54	.46		6	.091	.494	.331	13.9		
	3	.037	.066	.102	13.4				7	.089	.549	.048	10.3		
	4	.052	.153	.112	16.7				8	.012	.745	.030	18.4		
Biilmann's dimercurial(a)	1	.206	.516	.052	61.7			Baker's dimercurial(c)	1	.206	.517	.052	81.2		
	2	.146	.606	.008	36.8	.49	.46		2	.351	.411	.478	63.0		
	3	.184	.519	.458	19.1				3	.071	.786	.058	34.6	.57	.52
	4	.228	.581	-.002	15.3				4	.267	.560	.057	24.2		
									5	.384	.229	.076	20.1		
PAMA(b)	1	.198	.521	.052	36.6	.61	.48		6	.142	.801	.084	17.4		
	2	.222	.611	.000	6.9										
								K_2IrCl_6	1	.089	.387	.314	51.9	.48	.43

(a) $C_6H_{10}O_2Hg_2SO_4.H_2O$ (Ref.7) (b) p.acetoxymercurianiline (c) $[CH_3COOHgCH_2CH(OCH_3)]_2$

* Occupancy expressed in electrons and scaled by assuming full occupancy for the major Pb site in $Pb(CH_3COO)_2$.

\# $R_c = \sum \big| |F_{PH}| - |F_P| - |F_H| \big| / \sum |F_{PH}| - |F_P|$ for centric reflections.

ε is the lack-of-closure error of the phase triangle.

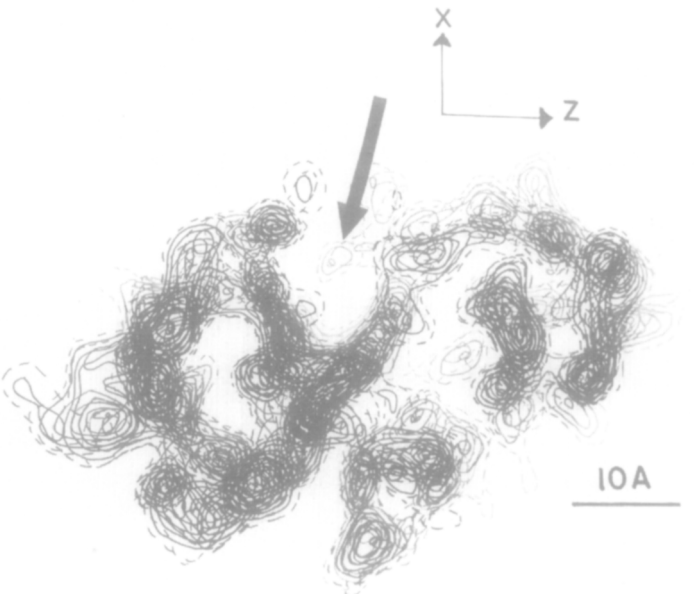

Figure 1a. Binding site cleft for epoxide and pepstatin molecules (arrow). The "β-barrel" structure is the left-half of the figure.

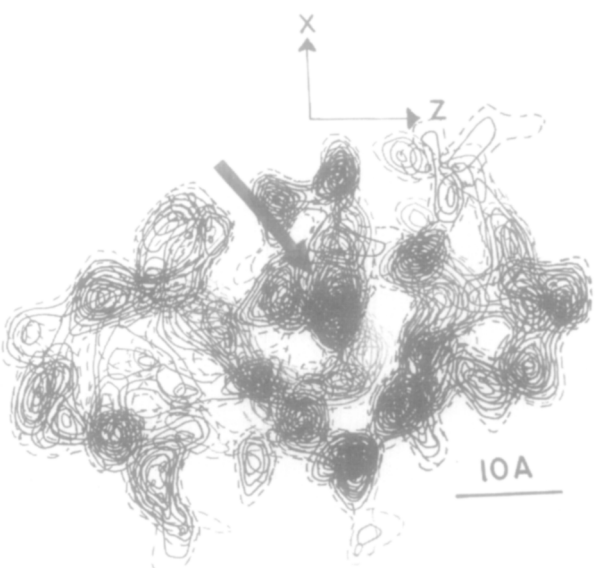

Figure 1b. Arrow indicates possible α-helical segment.

exhibits a striking distribution of electron density in the form of a hollow cylinder (Fig. 1a, left) running nearly parallel to the b axis of the crystal. The cylinder is located near x = 0.6a, z = 0.8c and has a length of about 30Å. It is elliptical in cross section with axes of 15Å and 10Å. Very similar features have been observed in the serine proteases (16,17), in the enzyme superoxide dismutase (18), in triose phosphate isomerase (22), in human plasma prealbumin (23) and in the immunoglobulins (19,20,21) where they have been shown at high resolution to be due to a structural arrangement known as a "β-barrel". The walls of the barrels consist of a number (6 to 8) of extended chains generally running anti-parallel to one another and forming a pleated sheet structure. Amino acid residues on the inside of the barrel constitute a predominantly hydrophobic core and do not in general show up as significant electron density at 5.5Å resolution.

The other lobe of the molecule is much less well defined. It contains one rod of high electron density also running roughly parallel to the y axis and located near x = 0.73, y = 1.20 to 1.40, z = 1.02. It is approximately 12Å long and could presumably represent a short piece of α-helix (Fig. 1b).

The structure is currently being extended to higher resolution. The availability of inhibitors together with the large amount of free space around the binding site suggests that this enzyme may be particularly favorable for further study.

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