THE CRYSTAL STRUCTURE OF PENICILLOPEPSIN AT 6 Å RESOLUTION

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SUMMARY: A 6 Å resolution electron density map of crystals of penicillopepsin, an acid protease from Penicillium janthinellum, has been computed from multiple isomorphous replacement phases determined from two heavy metal derivatives, K_2PtCl_6 and UO_2Cl_2. The mean figure of merit of the map is 0.939. The boundaries of the molecules, of which there are four per unit cell, are readily discernible. The molecule is highly asymmetric with approximate dimensions 60 Å x 40 Å x 30 Å. The molecule consists of two distinct lobes separated by a deep cleft, which is probably the extended substrate binding site.

Penicillopepsin is an extracellular acid protease produced by the mould Penicillium janthinellum. It resembles porcine pepsin and other acid proteases in amino acid composition, molecular weight, pH-optimum, specificity and reaction with covalent inhibitors (1-3). Recent studies on the catalytic mechanism have revealed a striking similarity between penicillopepsin and porcine pepsin (4). A comparison of the primary sequence of fragments of penicillopepsin with porcine pepsin provides evidence that the two enzymes are evolutionary homologues (5). It is hoped that the determination of the tertiary structure at high resolution will provide more compelling evidence for the evolutionary homology with pepsin the structure of which has been determined to 2.7 Å resolution (6). In addition, it should provide further insight into the complex mechanism of action of the acid proteases. As an intermediary step towards a high resolution structure of penicillopepsin we report the results of a 6 Å

resolution X-ray structure determination. Preliminary crystallographic data have already been published (7). Low resolution structures of two other fungal acid proteinases from Endothia parasitica (8) and Rhizopus chinensis (9) have recently been published.

Crystals of penicillopepsin were grown as described (7), except that in the present work the solvent used was 2.5 M Li_2SO_4 in 0.1 M sodium acetate buffer, pH = 4.4, instead of 2.5 M $(NH_4)_2SO_4$. The rhombic prismatic crystals are monoclinic, cell dimensions a = 97.4 (2) Å, b = 46.6 (1) Å, c = 65.4 (1) Å, β = 115.4 (2), space group C2.

Heavy-atom derivatives were prepared by soaking crystals in heavy-atom solutions made up with 2.5 M Li_2SO_4 at pH = 4.4. The soaking time required for up-take of heavy atoms was two weeks for 1 mM K_2PtCl_6 and one week for 1 mM UO_2Cl_2.

A Picker FACS-1 diffractometer, modified by an extended helium path for the diffracted beam collimator (crystal to counter distance 65 cm) was used to collect the intensity data (hkℓ and Friedel mate $h\bar{k}\ell$) within the 6 Å sphere. The incident radiation was Ni-filtered CuKα. The scan mode was θ/2θ with a scan speed of 2° min^{-1}, the scan width being 1.5° in 2θ. Individual background counts were measured at both limits of the scan for 10 sec each. Crystal damage due to X-irradiation was monitored by remeasuring three standard reflections every 50 reflections. The maximum variation of intensity of these three reflections was 4% over the whole range of data. With such a small apparent X-ray damage the complete set of data could be collected from a single crystal of the native enzyme and each derivative. A total of 1356 reflections were collected from each of the three crystals.

The agreement indices between Bijvoet pairs ($\Sigma \mid I_+ - I_- \mid / \Sigma\langle I\rangle$) was 0.037 for the reflections from the native crystal. The corresponding values for the K_2PtCl_6 and uranyl derivatives were 0.124 and 0.104 respectively. The agreement indices for the scaled structure factor amplitudes ($\Sigma \mid\mid F_{PH} \mid - \mid F_P \mid\mid / \Sigma \mid F_P \mid$) between the two derivatives

(K_2PtCl_6 and UO_2Cl_2) and the native crystals was 0.221 and 0.163 respectively.

Three dimensional difference Patterson maps were interpreted in terms of one major platinum binding site and three uranyl binding sites. Their y-coordinates were assigned by the use of Rossmann's function (10). The assignment of these coordinates was verified by calculating cross-phased Fourier maps (11).

Protein phases were calculated from derivative data by multiple isomorphous phase refinement in which heavy atom anomalous dispersion scattering data were included (12). Residual Fourier syntheses revealed two further minor platinum sites and one minor uranyl site. The refined heavy-atom parameters are given in Table 1. The overall figure of merit was 0.939. Refinement statistics for both derivatives are summarized in Table 2 and the determination of the absolute configuration from the anomalous data is given in Table 3. The R factors for both derivatives are satisfactorily small and give an important indication of the accuracy of the phase determination (13). It is noteworthy that the r.m.s. $|f_H|$'s are some four times higher than the corresponding r.m.s. errors, $|E_H|$ over the whole θ-range.

TABLE 1

Heavy atom coordinates ($x10^4$), relative occupancies, A, and temperature factors B($Å^2$)

Derivative	Site	x	y	z	A	B
$PtCl_6^{2-}$	1	571	(0)	169	28.0	43
	2	473	959	48	10.9	43
	3	598	165	83	8.4	43
UO_2^{2+}	1	286	565	239	14.4	29
	2	738	715	428	8.2	29
	3	337	777	347	9.4	29
	4	628	908	348	3.5	29

TABLE 2

Phase refinement statistics

Derivative	$PtCl_6^{=}$	UO_2^{+2}
R_k (all)	0.060	0.037
R_k (centric)	0.088	0.057
R_c (all)	0.255	0.249
R_c (centric)	0.260	0.259
r.m.s. $\lvert f_H \rvert$	60.86	39.41
r.m.s. $\lvert E_H \rvert$	14.39	9.12

$$R_k = \Sigma \left| F_{PH} - \left| F_P + f_H \right| \right| / \Sigma F_{PH}$$

$$R_c = \Sigma \left| \left| F_{PH} - F_P \right| - f_H \right| / \Sigma \left| F_{PH} - F_P \right|$$

where F_P, F_{PH} and f_H are structural amplitudes for native protein, derivative and heavy atom, respectively. $|E_H|$ is the lack of closure error in the phase triangles.

TABLE 3

Determination of absolute configuration

Anomalous Data	r.m.s.E_{Pt}	r.m.s.E_U	R_k(Pt)	R_k(U)	$\bar{m}$*
Included correctly	14.32	10.68	0.060	0.037	0.939
Included incorrectly	19.44	14.14	0.081	0.058	0.902

* $\bar{m}$ is mean figure of merit

The electron map was calculated with the use of the final 708 "best" phases (14) in sections perpendicular to the y-axis spaced about 2 Å apart.

In most areas the molecular boundaries were clearly defined. The molecule resembles an elongated and slightly flattened ellipsoid with the approximate dimensions of 60 Å x 40 Å x 30 Å. These dimensions are very similar to those reported for the acid proteinases of Endothia (8) and

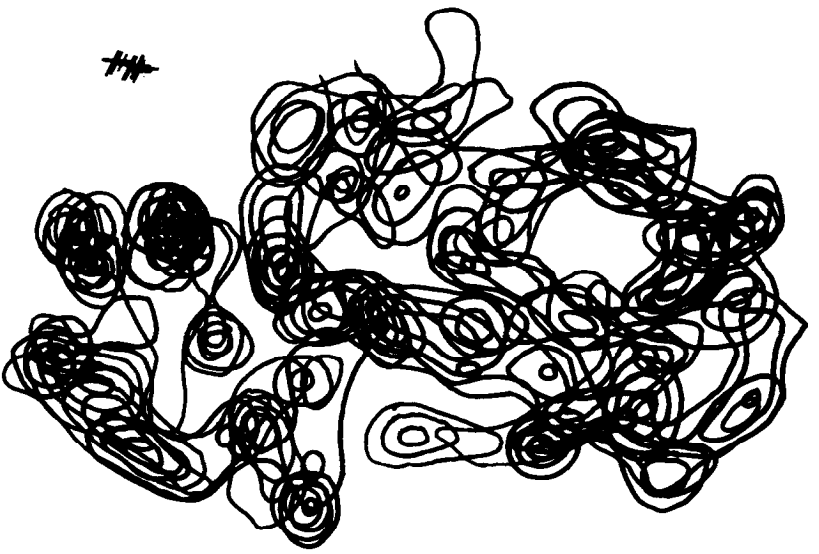

Fig. 1. Electron density map at 6 Å resolution viewed in a direction parallel to the b axis. Sections are calculated at 0.05 intervals between y = 0.10 and y = 0.40. The cleft which comprises the active site is at the bottom of this drawing of the map.

Rhizopus (9) and those of pig pepsin (6). Fig. 1 shows electron density maps representing one molecule. A deep groove running parallel to the 40 Å axis (b-axis) is clearly visible. Similar grooves have been reported for the Endothia protease (8), pig pepsin (15) and the Rhizopus protease (9). In the latter case it was shown that two active site inhibitors are located in the groove. It is therefore reasonable to assume that the groove in penicillopepsin contains the active site. It presumably also represents the location of the extended hydrophobic binding site, evidence for which has been obtained recently from kinetic studies with small substrates (Wang, T.T. & Hofmann, T., unpublished). This binding site is analogous to that proposed for pig pepsin (16, 17). The groove divides the molecule into two distinct compact globular regions.

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References

1. Sodek, J., and Hofmann, T. (1971) Meth. Enzymol. 19, 372-396.
2. Mains, G., Takahashi, M., Sodek, J., and Hofmann, T. (1971) Can. J. Biochem. 49, 1134-1149.
3. Mains, G., and Hofmann, T. (1974) Can. J. Biochem. 52, 1018-1023.
4. Takahashi, M., and Hofmann, T. (1975) Biochem. J. 147, 549-563.
5. Cunningham, A., Wang, H.-M., Jones, S.R., Kurosky, A., Rao, L., Harris, C.I., Rhee, S.-H. and Hofmann, T. (1976) Can. J. Biochem. (in press).
6. Andreeva, N.S., Fedorov, A.A., Gushina, A.E., Shutskever, N.E., Riskulov, R.R., and Vol'nova, T.V. (1976) Dokl. Akad. Nauk. SSSR, 228, 480.
7. Camerman, N., Hofmann, T., Jones, S., and Nyburg, S.C. (1969) J. Mol. Biol. 44, 569-570.
8. Jenkins, J.A., Blundell, T.L., Tickle, I.J., and Ungaretti, L. (1975) J. Mol. Biol. 99, 583-590.
9. Subramanian, E., Swan, I.D.A., and Davies, D.R. (1976) Biochem. Biophys. Res. Commun. 68, 875-880.
10. Stewart, J.M., Kundall, F.A., and Baldwin, J.C. (1970) X-Ray 70 System, University of Maryland, College Park, Maryland.
11. Rossmann, M.G. (1960) Acta Cryst., 13, 221-226.
12. Matthews, B.W. (1966) Acta Cryst., 20, 82-86.
13. Dickerson, R.E., Weinzierl, J.E. and Palmer, R.A. (1968) Acta Cryst., B24, 997-1003.
14. Blow, D.M., and Crick, F.H.C. (1959) Acta Cryst., 12, 794-802.
15. Andreeva, N.S., Borisov, U.U., Melik-Adamyan, U.R., Raiz, V.S., Trofimova, L.N., and Shutskever, N.E. (1971) Moleku. Biol. (USSR) 5, 908-916 (Engl. Transl. 5, 731).
16. Fruton, J.S. (1970) Adv. Enzymol., 33, 401-443.
17. Wang, T.T., and Hofmann, T. (1976) Biochem. J. 153, 691-699.