Structure of Acid Protease from *Endothia parasitica* in Cross-Linked Form at 2.45-Å Resolution†

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ABSTRACT: The strucure of acid protease from *Endothia parasitica* in strongly cross-linked form is compared with that of the untreated protein at 2.45-Å resolution. The only observed conformation change introduced by the cross-linking reaction is at the N terminal. Otherwise the two main chain structures are essentially identical. Approximately 2 molecules of the inhibitor, 1,2-epoxy-3-(*p*-nitrophenoxy)propane, are found to be incorporated into each protein molecule. They are covalently bound to the two aspartic residues at the active center.

In our previous reports on the structural studies of the strongly cross-linked acid protease from *Endothia parasitica*, we have demonstrated that the protein crystals could be cross-linked by glutaraldehyde in fairly concentrated ammonium sulfate solution (Wong et al., 1978a), and that such treated protein crystals are suitable for high-resolution structural determinations (Wong et al., 1978b). We have now improved the resolution to 2.45 Å in order to observe what conformational changes are introduced by the cross-linking reaction.

Experimental Section

Diffraction data on the three uranyl derivatives, UA, UB, and UC (Wong et al., 1978a), and on both the strongly cross-linked (CL) and untreated (NT) native crystals have been extended to 2.45-Å resolution. Friedel pairs were collected for all the uranyl derivatives. We used the data collection and processing techniques and the refinement procedures described in our previous report (Wong et al., 1978b). The statistics of refinement are listed in Table I.

Electron Density Maps. The best Fouriers (Blow & Crick, 1959) for both CL and NT crystals were calculated. The quality of the CL map can be illustrated by Figure 1, which shows a stereoview of 12 sections of the map in the region of the catalytic site and the extended substrate binding groove. The folding of the polypeptide chain, shown in Figure 2, did not differ much from the 3.5-Å result (Wong et al., 1978a). It is highly homologous to other known acid protease structures: *Rizopus chinensis* (Subramanian et al., 1977) and penicillopepsin (Hsu et al., 1977). It can be described as bilobal with the two lobes separated by a deep cleft. This protease is made mainly of mixed β sheets, consisting of 18 strands of polypeptide chains. There are 12 hairpin turns, all located on the surface of the molecule. The stretches of polypeptide chain that could be considered as helical are 231–233 and 180–183; they are all very short. The only disulfide bond observed in the CL map is between residues 263 and 288, similar to penicillopepsin (Hsu et a., 1977) and pig pepsin (Tang et al., 1973). Pig pepsin has two additional disulfide bonds, 45–50[1] and 206–210 (Tang et al., 1973). Although they are not cysteines, the corresponding residues of this acid protease (43–47 and 207–212, like penicillopepsin) are sufficiently close to be connected by a disulfide bridge.

The discontinuity of the main chain near the N terminal, mentioned in our last report (Wong et al., 1978b) as well as in the 3-Å investigation (Subramanian et al., 1977), is no longer observed in either the CL or NT map at 2.45-Å resolution.

In the CL map, the electron density bridges formed between neighboring molecules are clearly seen. Those bridges have lengths corresponding to either 5 or 10 carbons in a chain, as the glutaraldehyde is a mixture of monomer, dimer, and higher polymeric species (Richards & Knowles, 1968). Unlike the lightly cross-linked protein crystals, such as carboxypeptidase A in which only lysine residues seem to be modified (Quicho & Richards, 1966), the strongly cross-linked protein of the present investigation involves all the available >N–H and

Table I: Refinement Statistics at 2.45-Å Resolution[a]

	R_c		R_k		RMS(F_H)/RMS(E)		mean figure of merit, m		no. of reflections	
	CL	NT	CL	NT	CL	NT	CL	NT	CL	NT
UA	0.53	0.62	0.09	0.11	1.74	1.56				
UB	0.62	0.54	0.10	0.09	1.66	1.53	0.72	0.70	8276	7526
UC	0.52	0.62	0.09	0.11	2.03	1.72				
Pt[b]	0.55	0.63	0.09	0.13	1.64	0.94				

[a] $R_c = (\Sigma \| |F_{PH}| - |F_P| | - |F_H| |)/(\Sigma \| |F_{PH}| - |F_P| |)$; $R_k = (\Sigma \| |F_{PH}| - |F_P + F_H| |)/\Sigma |F_{PH}|$. R_c is summed over centric zone only; R_k is summed over the entire reflections; F_p is the structure amplitude for the native protein; F_{PH} is for the derivative protein; F_H is for heavy atom contribution alone; RMS is the root mean square; E is the lack of closure error of the phase triangle. For UA, UB, and UC, Friedel pairs were used in the refinement: $\Sigma \| |F_{CL}| - |F_{NT}| | / \Sigma |F_{CL}| = 0.1$; $\langle |\alpha_B(CL) - \alpha_B(NT)| \rangle = 40°$. [b] Pt extends to 5 Å only (Wong et al., 1978a).

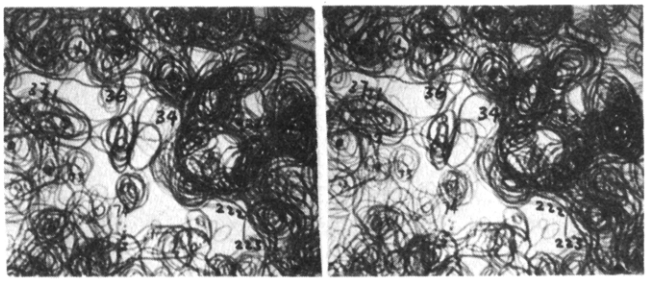

FIGURE 1: Stereoview of 12 sections of the CL map with intervals of 1 Å showing the active sites and the extended substrate binding groove. Black circles indicate approximate α-carbon positions for the respective residues. The carboxyl groups of Asp-33 and Asp-220 are hydrogen-bonded with each other. A cluster of three water molecules are hydrogen-bonded with residues 33, 220, and 222, and with each other. Tyr-74 can also be identified. NT and CL are nearly identical in this region including the cluster of water molecules.

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[1] Pepsin numbering (Tang et al., 1973) is in parentheses.

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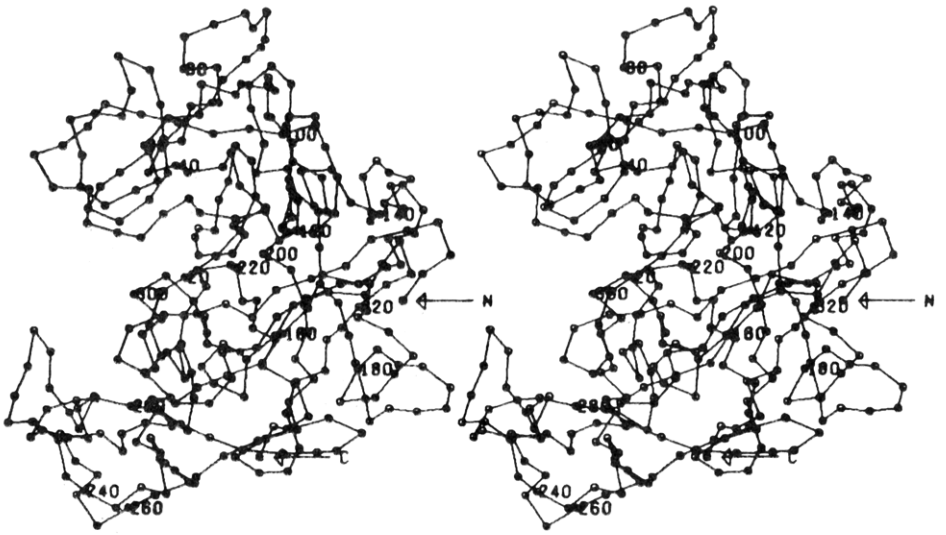

FIGURE 2: Stereo drawing of the α-carbon chain tracing for CL acid protease from *Endothia parasitica*. The numbers are temporarily assigned. They will be finalized when the sequence is completely known.

FIGURE 3: Stereoview of a cross-linking bridge between the NH_2 terminal and residue 283 of neighboring molecules. Residues 2 and 6 are also cross-linked.

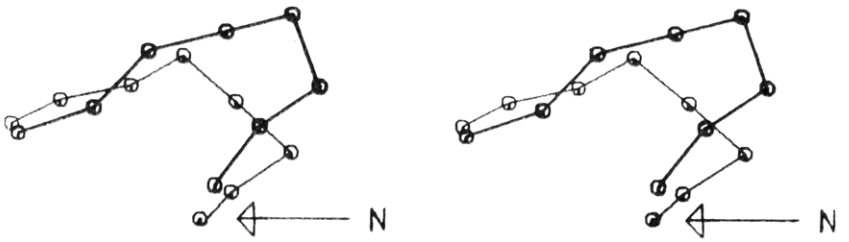

FIGURE 4: Stereo drawing of superposed CL and NT N terminals. Dark line is for CL, light line for NT.

$-NH_2$ groups (side groups and main chain as well) in the cross-linking reaction, provided that they are exposed to the solvent and that the distances between them are suitable. The cross-linking reaction could be produced by a glutaraldehyde oligomer reacting at one end by Michael addition and at the other end by reversible aldimine formation.

The $-NH_2$ terminal is cross-linked with residue 283 of the neighboring molecule (Figure 3). This reaction together with the intramolecular cross-linking between residues 2 and 6 causes conformation changes for the first 7 residues (Figure 4). This is the only significant conformation change observed.

Residues 43–46 and 107–109 are not observed in the 2.45-Å NT map, nor did they appear in the 3-Å model (Subramanian et al., 1977). Residues 43–46 are brought out in the CL map by an intramolecular cross-linking bridge between residue 44 and 87 (Figure 5). Likewise residues 107–109 are brought out by an intermolecular bridge between residue 108 and residue 261 of the neighboring molecule (Figure 6). These two features are also observed in *Rhizopus chinensis* (Subramanian et al., 1977) and penicillopepsin (Hsu et al., 1977).

The cross-linking bridges appearing in the CL map (Figures 4–6) in general have only one-half the electron density of the main chain. This is because they are probably disordered (formed by different species of glutaraldehyde oligomers) and very likely not fully occupied (as sites of heavy atoms).

Strong cross-linking reactions with glutaraldehyde have been widely used in preparing biological specimens for electron microscopic studies. Our result shows that the strong cross-linking reaction preserves the morphology of the biological specimens at the molecular level.

EPNP Binding to Protein Molecule. EPNP (1,2-epoxy-3-(*p*-nitrophenoxy)propane) is a specific inhibitor of acid protease and has been used to identify the residue Asp-32 at the active site for pig pepsin (Hartsuck & Tang, 1972).

We have successfully incorporated EPNP into both CL and NT crystals. The EPNP–enzyme CL crystals were made by soaking CL crystals in saturated EPNP solution (0.1 M EDTA)[2] in 0.01 M acetic buffer at pH 4.5 for 6 days at room temperature and at 4 °C, whereas the EPNP–enzyme NT crystals were prepared by soaking the NT crystals in saturated EPNP solution in original mother liquid (0.1 M EDTA) for 30 days at room temperature and at 4 °C. The 3.5-Å diffraction data for all the four types of EPNP–enzyme crystals (CL and NT at room temperature and 4 °C) were collected. Difference electron density maps were then summed using CL and NT protein phases (α_B), respectively.

Significant differences in density were found near the active sites for CL (room temperature and 4 °C) and NT (room temperature only). All three maps appeared very similar. They all can be interpreted as one EPNP is covalently bonded to Asp-220[3] (Asp-215) at the C_2 position in an extended

[2] Made into 0.1 M EDTA in order to remove any trace of heavy metal ions.

[3] Chemical modification studies with EPNP have shown that only aspartic acid residues are involved in covalent bonding with EPNP (Su, 1976).

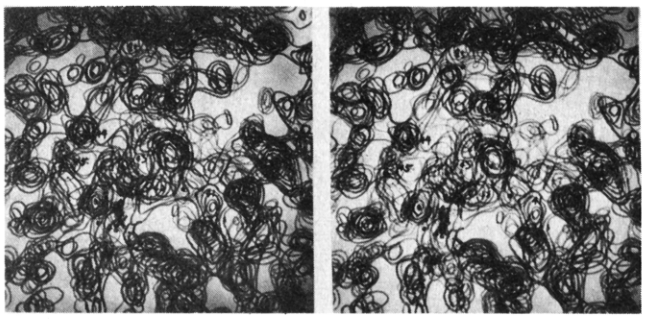

FIGURE 5: Stereo view of an intramolecular cross-linking bridge between residues 44 and 87.

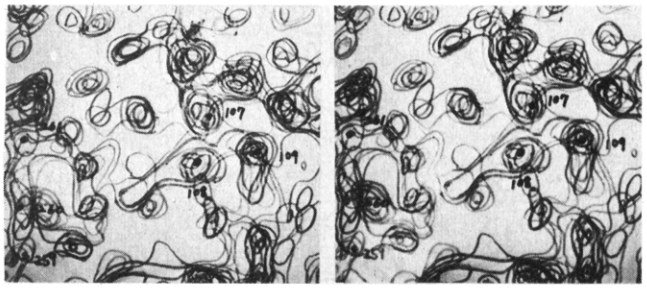

FIGURE 6: Stereoview of a cross-linking bridge between residue 108 and residue 261 of a neighboring molecule.

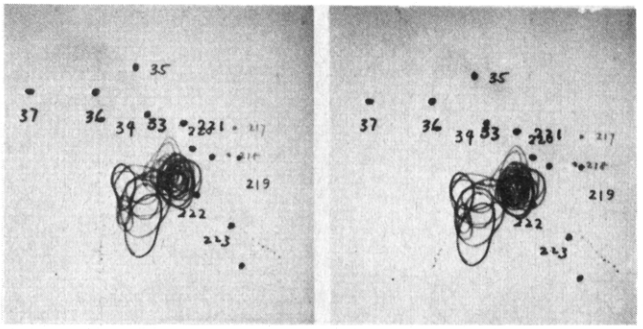

FIGURE 7: Stereoview of the difference map showing two EPNPs being in close contact. Both are in extended conformation. One is covalently bonded with Asp-220 at C_2 position and the other with Asp-33 at C_1 position. About half of the EPNP bonded to Asp-33 is obscured by the cluster of water molecules shown in Figure 1.

conformation with the long molecular axis of EPNP along *b*. The other EPNP is covalently bonded with Asp-33 (Asp-32) at the C_1 position with its long molecular axis also along *b*. The two EPNPs have a separation of 3.5 Å. About half of the EPNP bonded to Asp-33 is obscured by the cluster of water molecules shown in Figure 1. Figure 7 shows the difference density for the EPNP–enzyme CL crystal at room temperature.

Originally, the carboxyl groups of Asp-33 and Asp-220 are hydrogen bonded and share one electron as in the case in penicillopepsin (James et al., 1977 Figure 1). The way the two sites of EPNP molecules are bonded to their respective aspartic residues requires little conformation change for the aspartic residues as shown by the cleanness of the difference map (Figure 7). Also, residue 74 (Tyr-75) (probably a tyrosine also, see Figure 1 and later discussion) is not affected by EPNPs and need not make a conformation movement like that in penicillopepsin (James et al., 1977).

Chemical modification studies of this acid protease (Chen, 1977) show that modification of arginine by biacetyl results in reduction of activity by as much as 75%.[4] This suggests that residue 310 (Arg-308 in pepsin and Lys-308 in penicillopepsin) is probably an arginine.

Chemical modification by iodine results in complete loss of activity (Su, 1976). This is because residue 74 (Tyr-75) is iodinated resulting in a lower pK_a value and, therefore, cannot play its role properly in the catalytic mechanism (James et al., 1977). This result also suggests that residue 74 is a tyrosine.

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[4] Reduction of activity in cross-linked enzyme mentioned in our previous report (Wong et al., 1978a) is very likely partially caused by modification of arginine by glutaraldehyde.